THE EFFECT OF COCOA UPON CALCIUM UTILIZATION AND REQUIREMENTS, NITROGEN RETENTION AND FECAL COMPOSITION OF WOMEN [1]

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Wide interest has been evidenced in the effect of cocoa on the utilization of calcium, particularly that of milk, since Mueller and Cooney ('43) indicated that cocoa consumed with milk may depress the utilization of calcium in the growing rat by 20 to 25% when the proportion of milk solids to cocoa is only 3.3 to 1. Mitchell and Hamilton ('46) confirmed these results when the same brand of cocoa, "a low-cost American process cocoa containing 10.58% fat," was used. The present study was instigated to test the possible effect of a moderate-cost American process cocoa upon the calcium metabolism of humans, the group chosen for study being college women.

EXPERIMENTAL PROCEDURE

General plan

The study extended from October 10, 1944 to May 31, 1945 and was divided into 11 units during which several levels of

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cocoa were fed. Continuous calcium balances (broken only by 15 days for the Christmas holidays) were determined and the experiment was so planned that calcium utilization and requirement values could be calculated. During some of the later periods, nitrogen balances and fecal dry matter were determined.

Subjects

Seven college women, 18 to 22 years of age, served as experimental subjects during the initial part of the study. One withdrew at the Christmas vacation and was immediately replaced by an 8th subject, aged 20, who continued until the end of the study. These subjects were adjudged healthy as the result of an initial physical examination by a local physician. They ranged in weight from 47 to 72 kg and in standing height from 157 to 173 cm.

Dietary regime

(a) Composition of diets. A low-calcium basal diet containing an average of 225 mg of calcium and 70 to 80 gm of protein was fed. This diet consisted of 4 basic menus which were repeated in a definite order at 4-day intervals. The following foods were included: beef, ham, bacon, dried beef, gelatin, bread (70% extraction flour, milk-free), macaroni, rice, other cereals, potatoes, peas, corn, cabbage, lettuce, green beans, carrots, applesauce, peaches, apricots, pears, orange juice, tomato juice, grapefruit juice, butter, coffee, cream and sugar. The diet was supplemented daily by 5,000 U.S.P. units of vitamin A and 500 U.S.P. units of vitamin D in capsule form.[3] Caloric intakes were adjusted to individual needs with bread and sugar. Ad libitum consumption of distilled water was allowed. Whole homogenized milk[4] and cocoa powder were added in quantities enumerated in the following section. As these quantities were changed, adjustments were made in other

[3] Natola, Parke-Davis.

[4] A blend of three parts of whole and one part of skimmed milk was used in periods VIII and IX in order to adjust the fat content of the diet.

foods in an attempt to maintain a constant ratio of total calories, protein and fat. The cocoa used in this study was an American process cocoa of medium cost. The cocoa for the entire study was obtained in one lot at the beginning and by analysis had the following percentage composition: dry matter, 97.16; ether extract, 20.22; crude protein (N × 6.25), 22.56; tannic substances, 11.27; ash, 5.26; crude fiber, 3.50; theobromine, 1.79; phosphorus, 0.659; calcium, 0.145; oxalic acid, 0.504. It had an energy value of 5.48 cal. per gram.

(b) Feeding plan. The basal diet described above was used throughout. To this, foods were added in the order listed below during the 11 diet periods. The calcium content of the resulting diet is also shown.

Feeding plan for entire study

	ADDITIONS TO BASAL DIET	LENGTH OF PERIOD	CALCIUM INTAKE
			mg/day
I.	No additions	six 4-day tests	240
II.	Milk, 400 gm	six 4-day tests	670
III.	Cocoa, 5.6 gm; milk, 400 gm	five 4-day tests	679
	Christmas vacation (15 days)		
IV.	No additions	seven 4-day tests	226
V.	Cocoa, 58.2 gm; milk, 420 gm	three 4-day tests	724
VI.	Milk, 420 gm	four 4-day tests	684
VII.	Cocoa, 28.5 gm; milk, 420 gm	five 4-day tests	699
VIII.	Milk, 240 gm; vanilla ice cream,[8] 185 gm	six 4-day tests	755
IX.	Cocoa, 34.8 gm; milk, 240 gm; chocolate ice cream, 185 gm	three 4-day tests	752
X.	No additions	one 4-day and two 5-day tests	206
XI.	Cocoa, 21.0 gm	one 4-day and two 5-day tests	226

(c) Length of periods. In the initial plan of the experiment, the intention was to have a minimum of five 4-day tests in each dietary period. Period V, which lasted only 12 days, was ab-

[8] Ice creams for the study were made at the University dairy with the same basic formula for both the chocolate and the non-chocolate. A single lot of each was used throughout the ice cream periods.

ruptly terminated because of the adverse effects of the large amount of cocoa. Period VI was shortened, therefore, since 4 of the subjects had already had 12 days on the milk diet.

After the first 12 days of period IX, it appeared that the amount of cocoa being fed in conjunction with the diet containing 650 to 750 mg calcium per day had not affected the calcium balances, and it was decided to institute a more rigorous test by use of a low-calcium diet. The time remaining was then equally divided between periods X and XI.

(d) Reversal feeding. All subjects did not follow the exact schedule above, due to a reversal system of feeding. This system was instituted in an attempt to minimize the effects of changes in metabolism with time or with uncontrolled environmental conditions. In brief, it consisted of dividing the subjects into two groups, 4 and 3 subjects in each, one of which followed the order listed above and the other the following order: I, III, II, IV, VI, V, VII, IX, XI, and X. In this fashion, when a diet comparison was to be made each of the two diets to be compared was being fed simultaneously to two groups. It was intended to have a reversal of period V, but, as explained above, the plan was abandoned when the three subjects consuming cocoa reacted unfavorably to the large amount.

Sampling methods

(a) Foods. Aliquot portions of food were taken for analysis at the time of preparation. All foods were analyzed in 4-day pools. Bread and milk were each made into separate pools and the remaining foods composited. The food composites and milk pools were blended in a Waring Blendor and made up to a definite weight. They were preserved either with added acetic acid or by freezing until analyses were completed. Bread was prepared for sampling by drying at 110°C. and grinding.

(b) Excreta. Fecal periods were marked either by carmine or chromium oxide. The color of the carmine was difficult to detect in the feces of cocoa-fed subjects and the green color of the chromium oxide proved to be more easily detectable. Daily

urine samples and the fecal collections were composited into separate 4-day pools. The urines were preserved with either 1% hydrochloric acid or toluene. The fecal pools were blended with acetic acid and refrigerated until the analyses were completed.

Methods of assay

Calcium and dry matter were analyzed according to the methods of the Association of Official Agricultural Chemists ('40), with a slight modification in the calcium method. In this instance, alizarin-S red was used in place of methyl red as an indicator. Nitrogen was assayed by the Kjeldahl method and gross energy determinations were made in the Parr oxygen bomb calorimeter.

Supplementary observations

The physical well-being of the subjects was checked by daily weighings and by periodical physical examinations. Basal metabolism was determined by the gasometer technique (Boothby and Sandiford, '20) at the beginning of the study. Detailed records were made of unusual energy expenditures. No activity restrictions were invoked, however.

Estimation of calcium needs

Calcium utilization and requirement values were calculated from average calcium balance data.

(a) Utilization values were calculated according to the method of Breiter et al. ('41) by the following formula:

$$\text{Per cent utilization} = \frac{\text{Net losses}^{a} \text{ during basal period} - \text{net losses during test period}}{\text{Intake during test period} - \text{intake during basal period}} \times 100.$$

(b) Requirement values were determined according to the following formula (Outhouse et al., '41):

$$\text{Calcium requirement} = \text{Calcium intake} \pm \frac{\text{Calcium balance}}{\text{\% utilization of calcium}} \times 100.$$

[a] "Net losses" refers to the average daily calcium balance which, since the intake is below the estimated requirement, is negative, representing a loss.

PRESENTATION OF DATA

The data from these experiments are presented in tables 1 to 3. Table 1 shows the average daily calcium balance data of each subject for the 11 dietary periods. Average values for all of the subjects appear in the last column. Calcium utilization and requirement data for each subject are shown in table 2. Table 3 reports the average daily fecal dry matter and nitrogen excretion of individual subjects for periods V through XI. Caloric content of the feces for the same periods (excluding VI and VII) are also shown.

DISCUSSION AND RESULTS

The calcium balance data (see table 1) show considerable intra-individual variation. This was shown not only in the three periods (II, VI, and VIII) in which milk was added to the basal diet, but also in the three periods (I, IV, and X) in which the basal diet was fed alone. No consistent change in calcium balance was noted upon the inclusion of cocoa during any of the periods. The large amount of individual variation may account for the fact that no statistically significant difference was found between the average calcium balance observed on the cocoa and that on the non-cocoa diets. These averages for all subjects and all periods were -8.70 ± 9.7 [1] mg for the cocoa diets and -12.85 ± 11.0 mg for the non-cocoa diets, the difference being insignificant due to the high standard errors.

The extreme amount of individual variation among subjects and between periods for the same subject led the authors to an investigation of the degree of difference required between average calcium balances to show a significant effect of a given cocoa diet on calcium utilization. Analysis of variance of the average calcium balance values showed that a difference of 29, 57, 36 and 23 mg for periods II and III, VI and VII, VIII and IX, and X and XI, respectively, would be required in order

[1] In this instance and in all other cases, the value defining the significance of the mean is the standard error.

to demonstrate a dietary effect with a probability of 0.05. Because of this high degree of variance, the data do not preclude the possibility that an effect of cocoa upon calcium utilization may have been obscured by uncontrolled factors.

In comparing the change in calcium balance from one period to the following period, it was noted that 6 of the 7 subjects showed a decreased calcium retention in going from period II to III. A similar decrease was noted in all 7 subjects from period VIII to IX. This similarity of response to diet change for all subjects without regard to order of cocoa feeding would indicate a probable effect of time of feeding. These data emphasize the importance of a reversal feeding technique since, in the above instance, if all subjects had been fed the same diet simultaneously and had proceeded as a group to the second diet, the observed change in direction of retention might have been attributed to a specific dietary effect.

The extent to which the oxalic acid content of the cocoa may have contributed to the variation in calcium balances is discussed in a preliminary report of this study (Mitchell and Smith, '45).

Additional evidence of the similarity in value of the calcium of the cocoa and non-cocoa diets is shown by the calculated utilization values in table 2. In computing these values, the calcium balance during the basal period was an average balance for the three low-calcium periods. All milk periods were averaged for the non-cocoa diet evaluation, and for the cocoa diet evaluation all periods in which cocoa and milk were fed were averaged, regardless of the amount of cocoa involved. According to these data on utilization, the calcium of the cocoa diets was as well utilized as was that of the non-cocoa diets, the average utilization for the former being 21.3% as compared with 20.0% for the latter. The difference was insignificant statistically. These values fall within the lower portion of the range of 15.3 to 35.1% for 7 adults reported by Breiter et al. ('41). Steggerda and Mitchell ('46), in a study of the calcium utilization of 19 adult men, report values ranging from 16 to 45%, with

TABLE 1

Average calcium balance data, in miligrams per day, for 8 college women fed varying levels of calcium and cocoa

SUBJECT WEIGHT IN KG	A 72	B 46	C 58	D 62	E 47	F 58	G 68	H 50	AVERAGE
				Period I					
				Basal low calcium diet (six 4-day tests) Oct. 10–Nov. 2, 1944					
Intake	247.2	228.7	252.5	246.6	227.2	217.3	263.4	....	240.4
Fecal excretion	197.9	173.0	162.1	148.5	111.9	145.5	183.1	....	160.3
Urinary excretion	148.0	171.4	198.5	182.0	213.1	135.2	139.7	....	169.7
Balance	— 98.7	— 115.7	— 108.1	— 83.9	— 97.8	— 63.4	— 59.4	....	— 89.6
				Period II					
				Basal + 400 gm milk (six 4-day tests) Nov. 4–27, 1944					
Intake	671.3	667.5 [1]	669.6	680.7 [1]	667.6 [1]	646.8	683.5	....	669.6
Fecal excretion	493.1	524.8	413.2	433.9	382.5	460.9	497.5	....	458.0
Urinary excretion	179.2	192.0	244.1	289.2	295.3	145.6	187.4	....	219.0
Balance	— 1.0	— 49.3	+ 12.3	— 42.4	— 10.2	+ 40.3	— 1.4	....	— 7.4
				Period III					
				Basal + 400 gm milk + 5.6 gm cocoa (five 4-day tests) Nov. 28–Dec. 17, 1944					
Intake	688.7	667.0 [2]	688.7	679.3 [2]	667.1 [2]	662.4	701.9	....	679.3
Fecal excretion	561.8	534.5	482.6	434.7	381.8	494.2	502.8	....	484.6
Urinary excretion	171.0	154.7	228.9	268.2	273.1	156.2	186.3	....	205.5
Balance	— 44.1	— 22.2	— 22.8	— 23.6	+ 12.2	+ 12.0	+ 12.8	....	— 10.8
				Period IV					
				Basal low calcium diet (seven 4-day tests) Jan. 3–30, 1945					
Intake	242.8	221.0	232.4	232.7	224.2	213.7	....	215.8	226.1
Fecal excretion	187.1	172.2	160.0	134.3	109.8	148.4	....	149.5	151.6
Urinary excretion	144.6	136.9	197.7	231.6	207.4	140.6	....	120.2	168.4
Balance	— 88.9	— 88.1	— 125.3	— 133.2	— 93.0	— 75.3	....	— 53.9	— 93.9
				Period V					
				Basal + 420 gm milk for subjects A, D, E, H; basal + 420 gm milk + 58.2 gm cocoa for subjects B, C, F (three 4-day tests) Feb. 1–12, 1945					
Intake	688.5	....	....	686.8	672.0	....	....	671.5	....
Intake	....	702.7	742.4	....	....	727.1	....	....	....
Fecal excretion	531.6	507.3	492.2	395.9	373.0	623.4	....	420.2	....
Urinary excretion	179.1	141.6	223.2	302.9	276.7	178.2	....	194.5	....
Balance	— 22.2	+ 53.8	+ 27.0	— 12.0	+ 22.3	— 74.5	....	+ 56.8	....
				Period VI					
				Basal + 420 gm milk (four 4-day tests) Feb. 14–March 1, 1945					
Intake	689.1	681.2	709.2	688.1	676.4	666.5	....	675.5	....

Urinary excretion	179.1	141.6	223.2	302.9	276.7	178.2	....	194.5	....
Balance	— 22.3	+ 53.8	+ 27.0	— 12.0	+ 22.3	— 74.5	....	+ 56.8	....

Period VI

Basal + 420 gm milk (four 4-day tests) Feb. 14–March 1, 1945

Intake	689.3 [1]	681.2	700.8	689.3 [1]	672.1 [1]	683.0	....	672.1 [1]	684.0
Fecal excretion	510.7	508.2	606.1	422.9	402.8	502.6	....	475.7	489.9
Urinary excretion	187.0	165.5	216.4	248.6	252.5	176.5	....	135.9	197.5
Balance	— 8.4	+ 7.5	— 121.7	+ 17.8	+ 16.8	+ 3.9	....	+ 60.5	— 3.4

Period VII

Basal + 420 gm milk + 28.5 gm cocoa (five 4-day tests) March 3–22, 1945

Intake	717.9 [2]	677.1	698.3	718.4 [2]	700.1 [2]	677.1	....	702.2 [2]	698.7
Fecal excretion	611.2	512.5	613.5	478.2	420.2	568.5	....	530.2	533.5
Urinary excretion	141.0	146.7	169.2	223.5	232.5	139.2	....	152.0	172.0
Balance	— 34.3	+ 17.9	— 84.4	+ 16.7	+ 47.4	— 30.6	....	+ 20.0	— 6.8

Period VIII

Basal + 240 gm milk, cookies, candy, and ice cream (six 4-day tests) March 24–April 16, 1945

Intake	765.5 [1]	756.8	775.9	750.5 [1]	762.5 [1]	756.5	....	719.9 [1]	755.4
Fecal excretion	582.9	518.3	489.4	509.6	467.2	514.4	....	533.9	516.5
Urinary excretion	206.4	149.2	207.6	281.1	296.4	152.6	....	170.8	209.2
Balance	— 23.8	+ 89.3	+ 78.9	— 40.2	— 1.1	+ 89.5	....	+ 15.2	+ 29.7

Period IX

Basal + 240 gm milk + 34.8 gm cocoa in milk, cookies, candy and ice cream (three 4-day tests) April 18–29, 1945

Intake	765.3 [2]	747.8	759.8	757.3 [2]	748.0 [2]	745.8	....	741.4 [2]	752.2
Fecal excretion	599.7	536.4	556.4	550.5	463.2	601.4	....	513.6	545.9
Urinary excretion	132.1	129.8	166.7	224.8	246.4	141.6	....	119.0	165.8
Balance	+ 33.5	+ 81.6	+ 36.7	— 18.0	+ 38.4	+ 2.8	....	+ 108.8	+ 40.5

Period X

Basal low calcium diet (one 4-day and two 5-day tests) May 4–17, 1945

Intake	248.4	181.9 [1]		215.7	209.1	191.5 [1]	....	191.5 [1]	206.4
Fecal excretion	214.4	148.2		151.3	132.2	153.2	....	158.8	159.7
Urinary excretion	117.4	125.3	ill	149.5	136.0	115.5	....	115.8	126.6
Balance	— 83.4	— 91.6		— 85.1	— 59.1	— 77.2	....	— 83.1	— 79.9

Period XI

Basal low calcium diet + 21.0 gm cocoa (one 4-day and two 5-day tests) May 18–31, 1945

Intake	245.7	229.5 [2]		214.4	208.1	232.3 [2]	....	224.2 [2]	225.7
Fecal excretion	219.8	203.4		173.3	116.7	200.0	....	181.1	182.4
Urinary excretion	113.7	99.1	ill	138.9	126.6	89.9	....	87.3	109.2
Balance	— 87.8	— 73.0		— 97.8	— 35.2	— 57.6	....	— 44.2	— 65.9

[1] In the reversal feeding plan, these subjects consumed this diet during the time designated for the following period. Data were grouped thus for comparative purposes.

[2] Similarly, the data shown in this period were actually obtained at the time of the preceding period.

17 of the 19 values higher than any reported in the present study.

The average calcium requirement of the subjects when on the non-cocoa diet was 669 ± 42 mg daily, as compared with 686 ± 52 on the cocoa diet. Though 6 of the 8 subjects (see table 2) showed lower requirements when on the cocoa than on the non-cocoa diets, the differences for the entire cocoa and non-cocoa groups were not statistically significant. The overall average requirement of 678 ± 32 mg daily agrees with the

TABLE 2

Utilization of calcium from cocoa and non-cocoa diets, with individual calcium requirements calculated therefrom

SUBJECTS	NON-COCOA DIET		COCOA DIET	
	Utilization	Requirement	Utilization	Requirement
	%	*mg/day*	%	*mg/day*
A	16.7	787	17.6	756
B	23.3	634	26.9	577
C	22.5	761	22.0	772
D	17.3	813	17.8	765
E	19.1	657	23.9	568
F	23.9	509	19.9	931
G	13.8	694	16.5	649
H	23.3	498	25.6	470
Average	20.0	669	21.3	686

findings of Outhouse et al. ('41), who reported an average requirement of 662 mg daily for 7 adults. The requirement of 11.8 ± 0.5 mg/kg of body weight found in the present experiments is somewhat above the value of 10.7 mg/kg reported in the earlier investigation, the report of which also presents a comprehensive review of studies of calcium requirements published prior to 1941. Steggerda and Mitchell ('46), in the study mentioned above, indicate an average daily calcium requirement of 644 mg (S.D., 181), or 9.21 mg per kilogram body weight (S.D., 2.7) for the 19 men studied. Leverton and Marsh ('42) recommended a "minimal requirement" of 0.83 gm calcium per day for calcium equilibrium. This recommenda-

tion was made as the result of 100 calcium balance studies in college women on self-chosen diets, on the basis of the percentage of calcium retained at different levels of intake.

Although the calcium balances were not significantly altered when cocoa was removed from the diet, the division of calcium between urinary and fecal pathways was significantly affected. As may be seen in table 1, the fecal calcium fell and the urinary calcium rose with the removal of cocoa from the diet. Of the 27 cocoa comparisons made, 25 showed an increase in the urine calcium and 23 a decrease in the fecal calcium. Thus, the deviations from the ideal outcome of 13.5, if random factors alone operated, are 11.5 and 9.5, respectively. The standard deviation of 27 events which may occur in either one of two ways with equal probability is 2.6 ($S = \sqrt{27 \times .5 \times .5}$). Thus, the deviations from the ideal outcome are 3.5 or more times the standard deviation, and can be considered highly significant.

The extent of the increase in fecal calcium, when results on cocoa diets were compared with those on non-cocoa diets, was not directly related to the 4 levels of cocoa ingested. These increases were 26.7 ± 11.7, 43.6 ± 13.4, 29.4 ± 14.4 and 22.7 ± 10.6 mg calcium daily when 5.6, 28.5, 34.8 and 21.0 gm cocoa were fed. In a similar comparison of decreases in urinary calcium, the values were 13.5 ± 5.9, 25.5 ± 8.2, 43.4 ± 8.3 and 17.3 ± 4.3 mg when the 4 above-mentioned levels of cocoa were fed. Using the "t" test of Student ('25), it was found that both the increases in fecal calcium and the decreases in urinary calcium were statistically significant.

There is no doubt that there was a distinct effect of the cocoa upon the route of calcium excretion when cocoa was added to diets comparable in all other components. Although its over-all effect did not appear to change the availability of dietary calcium, as evidenced by the lack of differences in the calcium balance data, there must be some property of cocoa which physiologically affects the route of excretion of calcium. Factors which may affect urinary calcium excretion have been summarized by Knapp ('47). Calcium intake, considered by

this author as a most important factor, could scarcely have explained the data in the present paper. The acid-base balance of the diet, another factor discussed, though changed somewhat with cocoa feeding, did not appear to be sufficiently altered to account for the urinary decrease. Ash from 100 gm cocoa was found to require 32 ml normal acid for neutralization. The decrease in urinary nitrogen and calcium accompanying the feeding of cocoa may be suggestive of some specific physiological effect of the latter.

A study of nitrogen excretion was undertaken after the largest amount of cocoa had been fed. When this amount of cocoa (56.2 gm) was fed to subjects B, C, and F in period V, headaches, nausea and decreased appetites were reported. The subjective symptoms were accompanied by more frequent and voluminous stools. The stools were all well-formed and gave no evidence of a diarrheic effect of the diet. An investigation of the constituents of the feces was then undertaken in an attempt to determine the nature of the increase. This showed that the volume increase was accompanied by a similar increase in dry matter, which occurred in every case on inclusion of cocoa in the diet (see table 3). Analysis of variance showed that these increases in dry matter were not directly related to the amounts of cocoa fed.

The total gross energy value of the fecal excretion was also increased by cocoa feeding. This increase was in direct proportion to the dry matter excreted. No further analyses were made which would indicate the nature of the material which occasioned this increase in gross energy. The question arises as to whether undigested cocoa may have accounted for the change. With regard to caloric value, if none of the cocoa had been utilized, the caloric increase in the feces would have been twice that observed. When the gross energy per gram of fecal organic matter (dry matter — ash) was calculated, it was found that this value was lower on the cocoa than on the non-cocoa diet in 12 of 16 comparisons. These data suggest that the increased gross energy of the feces could not have been due to impaired fat digestibility.

TABLE 3

TABLE 3

Increase in average daily fecal excretion of dry matter, calories and nitrogen with cocoa feeding

DIET COMPARISONS	SUBJECTS						
	A	B	C	D	E	F	H
				Fecal dry matter (gm)			
Period V							
Milk diet	22.1	...	...	22.1	17.6	...	15.3
Milk diet + 58.2 gm cocoa	...	38.0	47.2	...	...	50.0	...
Periods VI and VII							
Milk diet	19.8	15.8	29.1	20.9	18.2	19.6	16.8
Milk diet + 28.5 gm cocoa	36.6	27.4	40.6	32.6	28.6	33.1	29.0
Periods VIII and IX							
Milk diet	20.6	14.7	25.4	21.3	19.1	20.0	16.0
Milk diet + 34.8 gm cocoa	32.2	27.9	36.8	29.9	27.1	28.6	23.1
Periods X and XI							
Low calcium diet	17.0	12.5	...	14.9	15.2	16.1	15.0
Low calcium diet + 21.0 gm cocoa	28.0	22.2	...	26.1	21.7	25.5	18.7
				Caloric value of feces			
Period V							
Milk diet	124.3	...	...	111.9	96.7	...	71.8
Milk diet + 58.2 gm cocoa	...	193.3	247.4	...	...	277.1	...
Periods VI and VII							
Milk diet	113.2	80.9	167.4	114.5	105.7	104.2	87.3
Milk diet + 28.5 gm cocoa	198.1	143.2	234.1	164.3	148.4	173.0	149.9
Periods X and XI							
Low calcium diet	105.6	76.5	...	88.8	88.0	92.7	82.1
Low calcium diet + 21.0 gm cocoa	168.2	137.1	...	147.6	119.9	147.7	111.4
				Fecal nitrogen (gm)			
Period V							
Milk diet	1.14	...	...	1.04	1.02	...	0.82
Milk diet + 58.2 gm cocoa	...	2.11	3.41	...	...	3.20	...
Periods VI and VII							
Milk diet	0.99	0.66	1.86	1.00	0.94	1.06	0.84
Milk diet + 28.5 gm cocoa	2.19	1.52	2.80	1.95	1.73	1.94	1.75
Periods VIII and IX							
Milk diet	0.90	0.61	1.52	1.10	0.88	0.96	0.72
Milk diet + 34.8 gm cocoa	1.94	1.62	2.61	2.04	1.81	2.11	1.39
Periods X and XI							
Low calcium diet	0.86	0.68	...	0.77	0.83	0.82	0.61
Low calcium diet + 21.0 gm cocoa	1.36	1.52	...	1.47	1.05	1.60	1.47

There was also an increase in fecal nitrogen with cocoa feeding (see table 3). This increase was no greater than the amount of cocoa nitrogen consumed, in 13 of 14 cases. In fact, additional fecal nitrogen amounted, on the average, to 84% of the extra nitrogen ingested on the cocoa diet. The increased fecal nitrogen shows a poorer digestibility of the protein in the diet when cocoa is included. A depression in the digestibility of protein has been noted by other investigators when cocoa was included in the diet. Cohn (1895) reported that 52.7% of the nitrogen remained undigested when cocoa was included in the mixed diet of human subjects. Neumann ('06), in studies with one subject, reported a decrease in the apparent digestibility of protein from 82.5% on a basic diet of bread, cheese and sausage to 75% on the same diet with 35 gm cocoa added. He attributed the loss of nitrogen to the increased fecal material caused by the cocoa, since he observed that the fecal nitrogen rose and fell with the amount of dry feces. Pincussohn ('07) also noted an unfavorable use of nitrogen by women consuming cocoa in a mixed diet. Lipman and Mueller ('41) reported a study on rats in which a decrease in the apparent digestibility of milk proteins from 85.3% to 79.3% occurred when 15.8% of American process cocoa was included in the diet. Mitchell et al. ('26) reported a true digestibility of 38% for cocoa nitrogen when fed to rats as the only source of nitrogen in the diet.

The increase in fecal nitrogen was accompanied by a decrease in urinary nitrogen so great that larger positive balances were observed with cocoa additions in 13 of 16 possible comparisons. The improved balances during the cocoa feeding were in most instances no greater than the increased nitrogen intake incident to cocoa feeding (two exceptions). In all instances where nitrogen balances were determined, they were positive whether cocoa was included or not. Neumann ('06), in the study cited above, also observed a decrease in urinary nitrogen along with an increase in fecal nitrogen. It is interesting to speculate as to whether the theobromine content of the cocoa may have accounted for the lowered urinary excre-

tion of nitrogen. Bernheim and Bernheim ('46) have shown that 0.3 gm of theobromine, given with each meal to two women who were not habitual drinkers of tea or coffee, caused a consistent decrease in the excretion of urinary nitrogen after the third day of administration. This same effect was noted in rabbits injected with doses of theobromine, although a tolerance gradually developed. Quantities of theobromine not unlike those used by the above workers were involved in the present study. The analyzed theobromine content of the cocoa was 1.79%; thus, when 56.2 gm of cocoa were fed, as in period V, the subjects were receiving 1.0 gm theobromine daily. Bernheim and Bernheim suggest an inhibition of urea formation in the liver as a cause of lessened urinary nitrogen, since they found that the total urinary nitrogen was accompanied by a decrease in urea nitrogen, with no consistent variation in the concentration of the other nitrogen compounds of the urine and decreases in urea and non-protein nitrogen of the blood. The nitrogen retention reported by Benedict ('16) after caffeine administration to one man suggests that this compound may also have exerted its effect by interference with urea formation.

SUMMARY AND CONCLUSIONS

The effect of the ingestion of a moderate-cost American process cocoa on calcium utilization was studied in 8 college women, 18 to 22 years of age. This involved estimation of calcium balances for 208 consecutive days, except for 15 days intervening for the Christmas holidays. Five levels of cocoa (5.6, 21.0, 28.5, 34.8 and 56.2 gm) were tested, 4 in diets containing milk as the major source of calcium and the other (21.0 gm) in a milk-free diet. Because of the bulky nature of the stools on the cocoa periods, additional observations were made on stool composition.

A summary of the results follows:

1. No statistically significant differences were demonstrated between the calcium balances of subjects on non-cocoa diets and those of the same subjects on cocoa diets. For all periods

and subjects the daily calcium balances averaged -12.85 ± 11.0 and -8.70 ± 9.7 mg for the non-cocoa and cocoa diets, respectively.

2. This similarity of response is further illustrated by the calculated calcium utilization values. For the 8 subjects, the latter values averaged $20.0 \pm 1.3\%$ on the non-cocoa diets and $21.3 \pm 1.4\%$ on the cocoa diets.

3. The average daily calcium requirements for all subjects were 686 ± 52 mg on cocoa and 669 ± 42 mg on non-cocoa diets. The average requirement for all subjects on all diets was then 678 ± 32 mg daily, or 11.8 ± 0.5 mg per kilogram of body weight.

4. Even though significant changes in calcium balance were not demonstrated to result from the addition of cocoa to the diet, there was a significant effect upon the path of calcium excretion. With the inclusion of cocoa, in 25 of 27 comparisons the urinary calcium fell, and in 23 of 27 cases the fecal calcium rose above that of adjacent periods in which cocoa was omitted.

5. With cocoa feeding, increases in the fecal excretion of dry matter and nitrogen were observed in each of 4 dietary comparisons. Total calories excreted increased in each of three possible comparisons.

It may be concluded that the tolerance of the subjects of this experiment for cocoa of the grade used was approximately one ounce daily, and that any possible deleterious effect of cocoa on calcium utilization was completely obscured by the variation (both within groups and within subjects) of the experimental data.

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THE EFFECT OF STORAGE ON THE NUTRITIONAL QUALITIES OF THE PROTEINS OF WHEAT, CORN AND SOYBEANS

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Grains are stored for various periods of time before consumption by farm animals or man. When there is a heavy carry-over from one crop year to another, or when the government attempts to stabilize grain prices by encouraging the storage of surplus grain, the storage periods may extend over many months. During such storage, changes in the chemical composition and nutritive value of the grain may occur. The nature and extent of these changes will depend upon the initial condition of the grain, particularly upon its moisture content, and upon the conditions of storage. Reviews of the literature relating to the chemical and nutritional changes occurring in grain during storage have been published by Jones and others ('43), Watson ('46), and Zeleny ('48).

Jones and co-workers have observed changes in the chemical properties of the proteins of corn (Jones, Divine and Gersdorff, '42), of wheat and wheat flour (Jones and Gersdorff, '41), and of soybeans (Jones and Gersdorff, '38), revealed by a decrease in solubility and a partial hydrolysis yielding free amino nitrogen. In all cases, digestion studies *in vitro* indicated a decreased digestibility of the proteins, and ad libitum feeding trials with stored corn revealed a diminished weight gain per gram of protein consumed, a change that might have resulted from a decrease in the palatability of stored, as compared with fresh, corn. The extent of the observed changes

depended upon the temperature and duration of storage, the type of container, and the nature of the material stored. Ground grains were less stable in storage than whole kernels. In the experience of Pickett ('48), the proteins of the peanut are quite stable during long periods of storage. Practical feeding trials with chickens have failed to indicate deterioration in the proteins of fish meals after short storage (Evans, Carver and Hamm, '44), while similar trials with pigs detected no deterioration in wheat stored for 4 years (Longwell, '45).

It may be noted that the corn and wheat samples studied by Jones and associates ranged in moisture content from 10 to 15%, on the borderline level, or below, for the inhibition of mold deterioration, according to Snow, Crichton and Wright ('44) and Milner and associates ('46, '47). Throughout the storage period of two years used by these workers, all samples remained free from any indication of insect infestation or mold infection.

More precise studies of the nutritional deterioration of food proteins during storage were reported by Henry and Kon ('48) using spray-dried skim milk of low moisture content, 3 to 7%, stored at room temperatures or higher. In rat metabolism studies, protein digestibility was slightly, if at all, impaired, except with powders containing 5% or more of moisture, but the biological value of the protein dropped from 86.9 to 65.9 during a storage period of 85 days at 37°C. The deterioration in protein quality seemed to relate almost entirely to lysine, since lysine supplementation corrected the damage. This is but another illustration of the sensitivity of milk proteins to environmental factors, shown by Fairbanks and Mitchell ('35) to obtain following brief exposures to high temperatures, as in spray drying. In this case, however, cystine was the point of attack. The changes in the essential amino acid content of the proteins of dry skim milk resulting from storage have been reported by Hodson and Krueger ('47), and by Henry, Kon, Lea and White ('48).

The experiments reported in this article were designed to study the effect on the utilization by growing rats of the proteins of wheat, corn and soybeans of storage for periods up to almost three years, under conditions that would favor continued respiration and enzyme action but would inhibit insect infestation and mold infection. The effects on the protein quality of corn produced by specific fungi in the field have been investigated by Mitchell and Beadles ('40) and by Mitchell, Beadles, Koehler and Dungan ('47). It will be shown that, under the chosen conditions, the proteins of corn and of wheat are resistant during storage to changes that would impair either their digestibility or their biological value. On the other hand, the proteins of the soybean undergo a considerable depression not only in digestibility but also in biological value.

EXPERIMENTAL PROCEDURES

The grains were all of the 1942 crop and the samples used in the experiment were obtained from the Department of Agronomy through the courtesy of Dr. George H. Dungan. They were fumigated before storage and were analyzed for proximate constituents, with the results shown in table 1.

Each of the grain samples was stored in two conditions, as whole kernels and as a meal ground to pass through a 1 mm sieve. The ground soybean sample was defatted and autoclaved for 90 minutes at 17 pounds steam pressure, to simulate commercial expeller-processed meal. The samples were stored in screw-top tin cans, capacity about one gallon, and were sealed with paraffin. Each container held enough sample for one biological assay with rats of the digestibility and biological value of protein. The cans, filled with whole or ground grains, were stored in a temperature-controlled room for periods up to about three years. Cans containing whole and ground samples of the same grain were withdrawn simultaneously and periodically for assay. The room temperature was maintained at about 78°F. or higher during extremely hot weather.

The experimental rations for the protein metabolism studies on growing rats contained salt mixture 446 (Spector, '48) 4%,

TABLE 1

Chemical description of samples

SAMPLE	DATE RECEIVED	DATE STORED	DATE OF FIRST TEST	PERCENTAGE COMPOSITION OF SAMPLES					
				Moisture	Protein (N × 6.25)	Ether extract	Ash	Crude fiber	Nitrogen-free extract
Wheat, Turkey Red variety	10– 2–42	10– 5–42	10–28–42	12.00	13.69	1.44	1.90	2.10	78.87
Soybeans, Illini variety	2– 9–43	3– 8–43	3–19–43	6.63 [1]	32.88	20.80	4.92	5.42	29.35
Corn, Station Yellow Dent no. 1	4–11–43	4–26–43	5– 6–43	9.23	9.88	4.47	1.27	1.91	73.24
Corn, U. S. Hybrid 13 [2]	5–11–43	6–21–43	6–12–43	11.56	8.19	4.30	1.23	1.94	72.78

[1] After defatting, autoclaving and dehydrating, these soybeans contained 6.17% moisture.

[2] (WF9 × 38–11) (Hy × L317).

NaCl 1%, fiber (wood flock)[1] 2%, wheat germ oil 0.5%, cod liver oil 1.5%, the test grain to the desired protein percentage, lard to 12% of total fat, sucrose 10% or less, and starch, or starch and glucose 1:1, to make up to 100%. The rations containing soybeans and wheat were made up to contain 10 to 12% protein ($N \times 6.25$), but the corn rations could not be made to contain more than 7 to 8% protein. The soybeans that were stored as whole kernels before incorporation into the experimental rations were defatted, autoclaved and dried in the same manner as the soybeans that were stored in a ground condition.

The digestibility and biological value of the test proteins were determined by the nitrogen metabolism method developed in this laboratory (Mitchell, '24a; Mitchell and Carman, '26), with some later modifications. These modifications include the use of a feces marker (Fe_2O_3); a reversal system of feeding whereby each rat receives samples of grain stored both as whole kernels and as a ground meal, in different experimental periods, 5 rats in one order and the other 5 in the reverse order; and a system of paired feeding which involves treating the 10 rats as 5 pairs for which the food intake is equalized in all periods. In each experiment, a sample of ground, dried and defatted beef round was tested along with the grains, generally by the same rats though at times in a separate test carried out simultaneously.

In the course of the two- to three-year experiment, several samples of beef were prepared and used, and all beef samples were stored in a sealed jar at 0°F. In each experiment, the determination of the metabolic fecal nitrogen per gram of food and of the minimum endogenous urinary nitrogen per $W^{\frac{2}{3}}_{gm}$ was made in a single centrally located period, or in an initial and a final period. All rations used in a single experiment were equalized in nitrogen, fiber and fat content and each experiment involved 10 to 15 weanling albino rats. The pre-

[1] Purchased from Brown Co., of Portland, Maine. Contains 94.3% cellulose, a trace of lignin and only 0.0033% nitrogen.

liminary periods in all experiments were from 4 to 7 days in length and the collection periods, 7 days.

The samples of soybeans, stored for 34 months, either whole or after grinding, defatting, and autoclaving, were tested for digestibility and the biological value of nitrogen, alone and when supplemented with methionine.

EXPERIMENTAL RESULTS

The average experimental results are summarized in table 2, including: (a) the digestibility of the protein with due allowance for the metabolic fecal nitrogen; (b) the biological value, representing the percentage of the absorbed nitrogen retained for maintenance (replacement of endogenous losses) and for growth; and (c) the net utilization, obtained by multiplying the digestion coefficients by the biological values and dividing by 100.

The standard errors of these average computations have not been given, because they are not necessarily valid in the comparison of samples taken at different times, and because they are of little use in the comparison of whole and ground grains sampled at the same time. The latter comparisons are best carried out according to the Student ('25) method of paired differences, since in each test grains stored whole and ground were generally fed to each of the test animals.

The pooled standard deviation of all individual coefficients of digestion,[2] computed from the sum of the squared deviations of individual coefficients about their respective group means, is 2.76. The corresponding standard error for the mean of a group of 10 is 0.87. The standard deviation of individual biological values, similarly computed, is 4.49, equivalent to a standard error of 1.4 for the mean of a group of 10. These latter two values may be compared with those reported earlier

[2] Omitting the coefficients of digestion for beef, since the variation of such coefficients is artificially minimized by the practice of considering all estimated coefficients above 100 as being equal to 100. The standard deviation of individual digestion coefficients of 2.76 is probably higher than what might be called normal because of the rather frequent occurrence of diarrhea in the experimental rats, especially in tests 334, 340, 344 and 346.

TABLE 2

Average results of the biological tests [1]

TEST NO.	STORAGE PERIOD	STORED AS WHOLE KERNELS			STORED IN GROUND CONDITION			BEEF ROUND STANDARD		
		True digestibility	Biological value	Net [2] utilization	True digestibility	Biological value	Net [2] utilization	True digestibility	Biological value	Net [2] utilization
	days	%	%	%	%	%	%	%	%	%
					Wheat					
318	0	88.80	49.1	43.6	88.80	49.1	43.6	99.80	72.4	72.3
331	354	89.18	53.5	45.5	85.88	50.6	43.5	98.25	76.9	75.7
348	730	88.40	46.2	40.8	89.80	46.5	41.8	99.50	79.2	78.8
					Soybeans					
325	0	85.30	69.1	58.9	85.30	69.1	57.3	99.30	73.3	72.8
334	256	78.30	62.6	49.0	85.70	73.7	63.2	99.44	78.6	78.1
344	404	80.00	66.1	52.9	84.67	69.2	58.6	98.70	75.3	74.3
371	1020	75.00	56.0	42.1	82.80	64.0	53.0	99.60	78.3	78.0
					Corn, Station Yellow Dent					
326	0	92.60	55.2	51.1	92.60	55.2	51.1	99.50	78.2	77.8
337	245	93.00	52.9	49.2	93.80	53.4	50.1	99.80	83.6	83.4
346	417	92.10	50.6	46.6	92.80	51.5	47.8	99.10	73.6	72.9
372	958	91.00	56.4	51.3	92.20	55.2	50.9	99.90	80.7	80.6
					Corn, U. S. Hybrid 13					
329	0	91.40	64.9	59.3	91.40	64.9	59.3	99.70	81.6	81.4
340	244	90.90	57.7	52.5	90.70	58.6	53.2	98.60	82.6	81.4
369	850	90.17	60.2	54.4	91.50	60.6	55.4	99.92	82.8	82.8

[1] With few exceptions, each value in this table is the average of values for 10 rats. In one experiment (331) the values given are averages for 8 rats, and in another (369) they are averages for 12 rats.

[2] Percentage digestibility multiplied by percentage biological value and divided by 100.

from this laboratory by Mitchell, Burroughs and Beadles ('36) of 3.7 and 1.2, respectively. The standard deviation of individual net utilization values, computed by the same method, is 4.44, equivalent to a standard error of 1.4 for the mean of a group of 10.

DISCUSSION

Changes in wheat and corn

The interpretation of the data in table 2 with reference to the main problem of the study, i.e., the effect of the storage of grains on the nutritive value of their proteins, should not be carried out on the basis of the intra-group standard deviations and the group mean standard errors, because these do not necessarily apply to inter-group variations when tests are conducted with different consignments of rats at widely different times. In assessing inter-group variation on diets similarly constituted, it was hoped that the results secured with the beef preparations, tested 14 times and at widely different intervals, would serve the purpose. However, as was explained above, the protein contents of the meat diets varied from 7.50 to 12.00 in accordance with the protein contents of the grain diets with which they were simultaneously tested. While this variation probably exerted no effect on the coefficients of digestibility, it would presumably modify the biological values. According to previous information secured in this laboratory (Mitchell, '24a; Mitchell and Beadles, '27), the relationship between biological values and the protein content of the diet is an inverse one. In the present case, the regression of biological value on protein content of diet is not linear, but of the following type: $\frac{x}{y} = -0.021 + 0.015x$, in which x is the percentage of protein ($N \times 6.25$) in the diet and y the associated biological value. The standard deviation of the observed mean biological values about the above regression line (i.e., the square root of the sum of squared deviations of observed from calculated y's divided by the number of degrees of freedom) is 3.02 (see table 3).

The standard deviation of the biological values of beef protein about the regression line relating biological values to

dietary protein levels, 3.02, may be used in detecting time changes in the biological values of the proteins of stored grains. An inspection of the biological values in table 2 suggests that, for stored wheat and stored corn, no time changes occurred. Such variations as occurred from one sampling to another were irregular; in particular, they reveal no consistent downward trend with lengthening time of storage, and for the Station Yellow Dent corn the final values are the same, or

TABLE 3

The relation between protein content of diet (x) and biological value of the protein of beef (y) as described by the equation: $\frac{x}{y} = -0.021 + 0.015x$

EXPERIMENT NUMBER	PROTEIN IN DIET	BIOLOGICAL VALUES		DIFFERENCES
		Observed	Calculated	
	%	%	%	%
331	12.00	76.9	75.5	−1.4
348	11.88	79.2	75.6	−3.6
318	11.62	72.4	75.8	+3.4
334	10.81	78.6	76.6	−2.0
325	9.94	74.5	77.6	+3.1
371	9.94	78.3	77.6	−0.7
344	9.69	75.3	77.9	+4.6
346	8.62	73.6	79.6	+6.0
337	8.50	83.6	79.8	−3.8
372	8.38	80.6	80.0	−0.6
326	8.31	78.2	80.2	+2.0
369	7.69	82.8	81.5	−1.3
340	7.59	82.6	81.8	−0.8
329	7.50	81.6	82.0	+0.4

somewhat higher, than the initial values. This impression is confirmed by the value for the pooled standard deviation of all mean biological values for corn and wheat samples about their respective sample means, i.e., 3.05. This standard deviation is almost identical with the standard deviation of mean biological values for the same protein mixture, that of beef, tested at different times through a period of two to three years, i.e., 3.02.

The variation to be expected from uncontrolled factors in the experiment affecting the digestibility of proteins cannot be assessed from the 14 mean coefficients of digestibility of

beef protein carried out at different times, because the digestibility of this protein ($N \times 6.25$) is so near 100 and because individual coefficients corrected for metabolic fecal nitrogen were entered as 100 whenever the estimation indicated a value over 100. Hence, resort must be had to the pooled standard deviation of individual values measured simultaneously about their respective group means.

The variation in average digestibility of the protein fraction of stored wheat and corn samples neither reveals nor suggests a depression of digestibility with lengthening time of storage. The standard error of a mean coefficient for a group of 10 rats tested simultaneously is 0.87, and that of a difference between the means of two such groups is 1.23. For an observed average depression in digestibility to be significant at the 2% level, assuming an improved digestibility on storage to be inconceivable in view of prior work indicating a reduction in protein solubility as storage progresses (Jones and Gersdorff, '41; Jones, Divine and Gersdorff, '42), the difference between means would need to exceed 2.47. Of the differences in mean digestibility of the protein for corn and wheat within any one series of tests, only two exceed 2.47, i.e., in the comparison of wheat, stored in the ground condition, in experiments 318 and 331, and 348 and 331. For these comparisons the variation in protein digestibility is irregular, with the highest coefficient obtained with the sample stored the longest.

The probable insignificance of all differences in mean digestibility from one sample of grain to another stored for a longer period is further indicated by the fact that the pooled standard deviation of the 20 mean digestion coefficients for corn and wheat samples about their respective group means, 1.04 ± 0.20, is not to be distinguished statistically from the standard error of a mean coefficient, 0.87, estimated from the variation among individual coefficients secured simultaneously.

The net utilization values for beef protein tested at 14 different times are correlated inversely with the protein content of the diet, as were the biological values, and for the same reason. In this case, the regression equation is $\frac{x}{y} = -0.022$

+ 0.015x, in which x is the precentage protein in the diet and y is the average net utilization of beef protein. The standard deviation of mean net utilization values about this regression line is 3.10. The standard error of a difference between two mean values would be 4.3. A difference between two means of 10 items each must be as large or larger than $4.3 \times 1.88 = 8.1$ to possess significance at the 2% level of probability. None of the differences between mean net utilizations for the same grain sample tested at different stages of storage is of this magnitude. For only the U. S. Hybrid corn sample is there any suspicion that storage may affect protein utilization, and here the difference between the values for the initial sample

TABLE 4

Differences in the mean digestibility and biological values of corn and wheat stored as kernels or as ground meal [1]

	GRAINS STORED WHOLE	GRAINS STORED GROUND	DIFFERENCES
Digestibility	90.25 ± 0.54	90.85 ± 0.22	0.60 ± 0.59
Biological value	53.71 ± 0.39	53.99 ± 0.26	0.28 ± 0.47
Net utilization	48.56 ± 0.45	49.10 ± 0.27	0.54 ± 0.52

[1] The means are given with their standard errors and were obtained by pooling the results of 7 samplings of stored wheat and corn.

and for the final samples after 850 days of storage is 4.9 percentage units for the samples stored whole, and 3.9 percentage units for the samples stored as a ground meal.

It is quite commonly supposed that grains keep better when stored as whole kernels than when stored as ground meals. For the corn and wheat samples studied in this investigation, and with reference to protein utilization by growing rats, this was not in general true. The statistics given in table 4 were secured by comparing whole and ground grains without reference to pairing. The differences between stored whole grains and stored ground meals in mean digestibility, biological value and net utilization of nitrogen are evidently insignificant statistically.

Paired comparisons were made in those experiments in which the mean digestion coefficients or biological values differed to such an extent as to suggest a difference between kernel samples and ground samples, i.e., in experiments 337, 346, 348, 369 and 372. In these comparisons, the differences in digestibilities or biological values for each rat on stored whole sample and on stored ground sample were analyzed by the Student ('25) method. None of the differences proved to be highly significant ($P = 0.02$ or less), though one of them approached significance. In experiment 348, involving wheat samples stored for 730 days, the mean digestibility of nitrogen in the whole grain storage sample was 88.40, and that in the ground grain storage sample was 89.80%. The "t" value of the difference is 2.28 and the probability that a random combination of the uncontrolled factors in the experiment would have produced a difference, in the same direction, as great or greater than this, is 0.024. It is to be noted that, in this case, the nitrogen of the ground sample exhibited the higher average digestibility. The other probabilities obtained in these paired comparisons ranged from 0.14 to 0.29.

It is worthy of note that the biological values for whole wheat protein recorded in table 2, averaging 49.2, are less than the average of 67 previously reported from this laboratory (Mitchell and Carman, '24) for wheat of unrecorded variety. In unpublished experiments we have since found a biological value of 43.4 for the proteins of whole wheat flour from durum wheat. There is more than a suspicion on the basis of these findings that there are marked differences among different varieties of wheat in the nutritional value of their proteins. The proteins of the open-pollinated variety of corn exhibited an average biological value of 53.6 and those for the hybrid variety, 61.1. These values agree well with the value of 60 previously reported for corn (Mitchell, '24b).

Changes in soybeans

The soybeans stored in a ground condition, preheated and defatted, varied in protein digestibility and biological value

from sample to sample in an irregular fashion. The changes in protein utilization were too small to be considered significant, except for the changes that occurred from 404 days of storage to 1,020 days. In this interval, the protein digestibility dropped from 84.67 to 82.80, a drop of 1.87; the biological value dropped from 69.2 to 64.0, a difference of 5.2; and the net utilization from 58.6 to 53.0, a decrease of 5.6. Judged by the criteria used in the interpretation of the values secured with the wheat and corn samples, these differences are suggestive of storage effects but do not constitute conclusive evidence. The drop in digestibility of 1.87 is less than the critical difference of 2.47 between means of 10 variates. The drop in biological value of 5.2 is only slightly more than the standard error of a difference between the means of two groups of 10 variates sampled at different times, 4.3 [$\sqrt{(3.02)^2 + (3.02)^2} = 4.3$]; while the drop of 5.6 in net utilization is much less than twice the standard error of a difference between two means of 10 variates each, i.e., 4.3.

The soybeans stored whole, however, tell a different story. For 404 days of storage, the changes in biological value in these samples were irregular and not clearly indicative of an effect of storage. The fall in digestibility during this period from 85.30 to 80.00 is clearly a real change if judged by the critical value of 2.76 used in the analysis of the wheat and corn data. While this value is based upon intra-group variation, for reasons given above, it seems to be fairly applicable to the variation of means of samples taken at different times.

Between 404 and 1,020 days of storage the average digestibility of the soybeans stored whole dropped 5.00 percentage units, the mean biological value dropped 10.1 percentage units, and the mean net utilization dropped 10.8 percentage units. The reality of these decreases in protein utilization seems clear by the criteria of significance developed in the preceding section.

The sample of soybeans stored in the unground condition may be readily compared with respect to utilization of protein with the sample preheated and stored in the ground condition,

since in each of the three tests each experimental rat received both types of bean samples. In protein digestibility, the soybeans preheated and stored as a defatted meal exceeded the soybeans stored whole and unheated by 7.40 percentage units at 256 days of storage, by 4.67 precentage units at 404 days of storage, and by 7.80 percentage units at 1,020 days. In each case all paired comparisons favored the beans heated before storage, so the statistical significance of the differences is clearly established.

A similar comparison of biological values favored the samples heated prior to storage, the average differences being 11.1, 3.1 and 8.0 percentage units at 256, 404 and 1,020 days of storage, respectively. Statistical analysis of the individual paired data showed the first difference to be highly significant, data from all rats agreeing in the verdict. The biological values in the second test (404 days of storage) were so variable that no significance can be attached to the average difference. At 1,020 days' storage, the difference in biological value was significant at the 5% level ($P = 0.050$).

The whole picture supports strongly the conclusion that soybeans stored at 75° to 80°F. without pretreatment undergo changes in chemical composition that induce a marked reduction in the digestibility of their nitrogen and in its biological value for the growing rat. These changes are not brought about by insect infestation or by any observed mold infection, and they occur in beans containing less than 10% of moisture. Under the conditions of this investigation, the impairment in nitrogen digestibility averaged 10 percentage units in 1,020 days of storage, and the impairment in biological value averaged 13 percentage units. The total impairment in the nutritive value of nitrogen, as measured by the net utilization values, amounted to about 17 percentage units, representing a decrease of 28%. This depression in the nutritive value of the proteins of soybeans on storage is largely prevented by pretreatment with heat, 90 minutes at 17 pounds steam pressure. The grinding and defatting of the beans prior to autoclaving presumably were minor factors, if they operated at all, in these changes.

The conclusion that grinding prior to storage is a minor factor in protein preservation under conditions that eliminate the action of insects and molds is based upon the information secured with corn and wheat.

Soybean proteins are deficient nutritionally in the sulfur-containing amino acids. This fact has been demonstrated by Mitchell and Smuts ('32), Shrewsbury and Bratzler ('33), and many others. This is also true of the heated soybean (Mitchell, Hamilton and Beadles, '45). The effect of supplementing with methionine the protein in the final samples of soybeans, stored either whole or ground but in both cases autoclaved before testing, was measured by the nitrogen balance technic previously described. The average results for this test are summarized in table 5.

From these data it is evident that methionine supplementation had no demonstrated effect upon nitrogen digestibility in either sample of soybeans, but that it exerted a definite effect in raising the biological value of the nitrogen in each sample. The favorable effect of methionine supplementation was much greater for the beans heated before storage than for those not so treated. In the first case, the average biological value was increased by 20.2 percentage units, from 58.3 to 78.5; in the case of the beans stored whole, the biological value was raised only 6.0 percentage units, from 54.0 to 60.0. Both increases were highly significant statistically.

Since methionine supplementation improved the biological value of the proteins in both storage samples of soybeans, and since methionine (or cystine) effectively supplements soybeans, raw or heated, that have not been stored (Mitchell, Hamilton and Beadles, '45), it follows that the damage inflicted by storage on the metabolic utilization of soybeans is due primarily to an effect upon the cystine or methionine (or both) contained in the soybean protein. The fact that methionine supplementation improved the biological value of the preheated soybeans, after storage, much more than that of the

untreated soybeans stored as whole beans, indicates that the greater storage damage in the latter case involved also other amino acids than cystine and methionine.

TABLE 5

Average results of supplementing with methionine the proteins of soybeans stored ground and as whole kernels for 34 months. (Each value relates to a group of 6 growing rats)

	TRUE DIGESTIBILITY	BIOLOGICAL VALUE
	%	%
I. Soybeans stored as a defatted autoclaved meal for 34 months		
No methionine supplement	83.8	58.3
With methionine supplement	85.7	78.5
mean differences	+ 1.8	+ 20.2
standard deviation	3.97	8.73
probability	0.18	0.0017
II. Soybeans stored as whole kernels for 34 months		
No methionine supplement	77.2	54.0
With methionine supplement	76.8	60.0
mean differences	— 0.33	+ 6.0
standard deviation	2.16	2.10
probability	0.37	ca. 0.0005

Interpretations

The results of the experiments on corn and wheat, indicating a high resistance of cereal proteins to nutritional change by agents that operate during storage at a low moisture level, are in agreement with the findings of Robertson, Lute and Gardner ('39) that cereals (wheat, oats and barley) stored at a moisture level of 10% may be expected to last for two years or more if in good condition initially. In fact, they may retain 80% of their original ability to germinate after 15 years' storage (Robertson and Lute, '37). As Oxley ('48) has pointed out, ". . . germination is the most sensitive characteristic of grain and hence it is generally true to say that if the germination is good, the grain is completely undamaged from the point of view of milling."

In another connection Oxley says, "Cereal grains are seeds, that is, they are the dormant resting stage of the plant which bears them. Though they are alive, and all living things are continually respiring, producing heat, water, and carbon dioxide, they are at a very low ebb. It is the combined effect of their continued life (which enables them to resist decomposition by micro-organisms) and the very low level of that life, which makes cereal grains such pre-eminently stable bodies in store."

The results obtained with cereal grains are not necessarily opposed to the findings of Jones and co-workers ('41, '42), since the changes in solubility of the proteins in saline solutions and the partial proteolysis observed by these workers in stored grains do not necessarily imply a change in the nutritive value of the proteins. The decreased digestibility noted in tests carried out *in vitro* refers to rate of digestion rather than to the completeness of digestion, while the decreased growth-promoting power reported for the proteins of stored corn in ad libitum feeding trials may have been brought about solely by a diminished food consumption conditioned by an impairment in the palatability of the corn during storage.

The marked detrimental effect of prolonged storage on the nutritive properties of the soybean, when the bean is not subjected to pre-treatment other than fumigation, places the soybean in marked contrast to the cereal grains. The possible causes for this distinction may rest largely in the difference in anatomical structure. The living part of the seed is the embryo or germ. The embryo accounts for only 2% or less of the wheat kernel and 9 to 10% of the corn kernel, but in the soybean it accounts for 92% of the bean, which consists entirely of seed coat and embryo. Embryonic respiration would be expected to be more intense in the soybean, therefore, than in corn or wheat seed. The soybean contains a wide variety of enzymes (Waksman and Davison, '26), some of which may attack the amino acids produced from the seed proteins through protease action. The fact that preheating of soybeans largely eliminates the detrimental effect of storage

on the nutritional quality of the proteins is in harmony with the view that the reactions involved are to a large extent enzymic in nature.

Another possible cause of protein deterioration in soybeans in storage is the occurrence of reactions between the proteins or the proteinogenous amino acids and the sugars, mainly pentoses, that occur in soybeans to the extent of about 10% (MacMasters, Woodruff and Klaas, '41). This sugar-amino acid reaction, the so-called "browning reaction," has been shown by Henry, Kon, Lea and White ('48) to account for the nutritive deterioration of the proteins of dried skim milk in storage at moisture levels of 5 to 7%, the point of attack being the ε-amino group of lysine in the intact protein. Glycinin, the globulin of the soybean, has been shown to react in the same way on refluxing with glucose (Patton, Hill and Foreman, '48). The amino acids which seemed to be attacked by "browning" an intact protein are chiefly those containing functional nitrogen groups unattached in peptide linkages: epsilon amino (lysine), guanido (arginine), indole (tryptophan) and imidazole (histidine) groups. However, amino acids liberated from soybean proteins by the proteases present would be subject, presumably, to the same reaction. This is not an enzymic reaction, but it is one that is accelerated by heat; it would therefore occur both in the soybean sample stored whole and then autoclaved before being tested and in the sample autoclaved before storage. The smaller indicated protein impairment in the latter case may be a result of the "browning reaction" only.

SUMMARY AND CONCLUSIONS

The effect of storage at approximately 78°F. (25.5°C.) and at moisture contents of 6 to 12% on the nutritive value of the proteins of wheat (Turkey Red), corn (an open-pollinated and a hybrid variety) and soybeans (Illini) was determined. The storage periods lasted from 730 to 1,020 days. The corn and wheat samples were stored both as whole kernels and as meals ground to pass a 1 mm sieve. The soybeans were stored

as whole beans and as an autoclaved, defatted, and dehydrated meal. The beans stored whole were autoclaved, defatted, dehydrated and ground in the same manner before being tested.

The tests of the biological availability of the proteins ($N \times 6.25$) of the stored seeds were carried out two or three times during the storage period by the nitrogen balance procedure developed in this laboratory, using growing albino rats as subjects. The utilization of the seed proteins in digestion was measured by the coefficient of true digestibility, making due allowances for the non-dietary nitrogen (metabolic nitrogen) in the feces. The utilization in metabolism was measured by the biological value, representing the percentage of the absorbed nitrogen retained for maintenance and for growth under standardized conditions of feeding.

The methods of testing used detected no consistent or significant deterioration in the digestibility or the biological value of corn and wheat proteins during storage for two to three years, either as whole kernels or as a ground meal.

Soybeans stored for 1,020 days as raw whole beans deteriorated definitely and significantly in both the digestibility and the biological value of their nitrogen (protein). Under the conditions of storage imposed, the average impairment in digestibility of nitrogen amounted to 10 percentage units, while the impairment in biological value averaged 13 percentage units. The total impairment in the nutritive value of nitrogen, as measured by the net utilization values, amounted to about 17 percentage units, representing a deterioration in the nutritive value of conventional protein ($N \times 6.25$) of 28%. Since the preheating of soybeans prior to storage prevented this deterioration largely or entirely, the reactions responsible for observed changes are probably largely enzymic in character and involved in the respiration of the seed embryo, which makes up 92% of the whole seed. The possibility of sugar-amino acid involvement is discussed.

Working with samples stored for 1,020 days, methionine supplmentation improved the biological value of both storage samples of soybeans, as it does that of raw or heated freshly

harvested beans; in the case of the samples stored raw and as whole beans, the average increase in biological value amounted to 6 percentage units, while for the preheated sample the increase averaged 20 percentage units. These facts permit the conclusion, first, that the damage inflicted by storage on the metabolic utilization of soybean proteins relates primarily to the cystine or methionine (or both) components of soybean proteins; and, second, that the greater storage damage in the unheated beans involves also other amino acids than cystine or methionine.

From the results of these and other studies it appears that the nutritive value of the proteins of cereal grains stored under conditions preventing insect infestation and mold growth (by lowering moisture content below a critical level) is not appreciably altered by seed respiration over long periods of time. This is not true, however, for the soybean, the proteins of which may suffer considerable nutritional damage after one year of storage. The distinction between the two types of seed may reside largely in the fact that the living part of the seed, the embryo, constitutes only a small part of the cereal seed but a predominant part of the legume seed.

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A GROWTH FACTOR TRANSMITTED BY THE HEN THROUGH THE EGG TO HER PROGENY [1]

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That the cystine and methionine content of a hen's egg can be increased by changing the hen's diet from a ration in which corn is the chief source of protein to a high protein ration containing 20% casein or 20% soybean protein has been shown (Csonka et al., '47).

This study was undertaken to learn whether or not there would be any difference in the growth of chicks hatched from these eggs. Chicks from the eggs of hens fed high- and low-protein diets were reared together and fed identical rations. From our observations of these chicks, we conclude that the statistically significant differences in growth were not related directly to the increase in the cystine and methionine content of the eggs but were due solely to differences in their mothers' dietary proteins, the chief variable component in our study.

EXPERIMENTAL PROCEDURE

We carried out two series of experiments, one in 1947 and the other in 1948. In each series, two pens of crossbred pullets in their first laying season were used. Each pen contained from

[1] This is the first article in a series dealing with the influence of dietary protein. A preliminary report was presented by the authors before The Federation of American Societies for Experimental Biology, 1949 annual meeting, held at Detroit, Michigan. Federation Proceedings, Part 1, Volume 8, page 193 (1949).

15 to 18 hens and one rooster. The chickens in each pen were fed rations differing in quantity and quality of protein.

The basal, low-protein diet (table 1) contained all the known nutrients in sufficient amounts for egg production and good hatchability, including approximately 9 gm of crude protein supplied mainly by yellow corn. The two high-protein diets were identical to the basal diet except that in one 20 parts of the corn meal were replaced by casein (commercial), and in the other 50 parts of the corn meal were replaced by soybean meal (commercial).

TABLE 1

Composition of the basal diets fed to laying hens and to their chicks

INGREDIENTS	HEN FEED (low-protein)	CHICK MASH
Yellow corn	87	50
Alfalfa leaf meal	5	12
Pulverized oats	..	30
Wheat meal	..	47
Soybean meal	..	47.5
Sardine meal	..	6
Limestone flour	2	2.5
B-Y feed (250 μg riboflavin per gram)	0.5	0.92
Manganized salt (96% NaCl; 6% $MnSO_4$)	0.5	1.0
Iodized salt	0.2	..
D-sterol	..	0.075
Vitamins A and D feeding oil (2,000 I.U. of vitamin A and 400 A.O.A.C. units of D per gram)	0.3	..
Steamed bone meal	4.5	..

In each of the 9 experiments (table 2), hens in the two pens were fed simultaneously and their eggs collected for incubation. For instance, in experiment 1 a low-protein ration was fed to the hens in pen 1 and a high-protein ration containing casein to those in pen 2. In experiment 2 a similar feed was given to the same hens. In experiment 3 the rations were changed; hens that had been on the low-protein diet were put on a high-protein ration containing soybean meal, and those previously on a high-protein diet containing casein were fed a low-protein ration, plus dried cow manure.

This procedure for simultaneous feeding of the hens in the two pens and for changing the diets at specified intervals was continued in experiments 4, 5, 6, 7, 8, and 9 (1948). Whenever a hen's diet was changed, a period of three weeks was allowed to elapse before her eggs were used. This was done to eliminate the dietary effects of the previous ration on the eggs produced.

TABLE 2

Various diets and the order in which they were fed to the hens whose eggs were collected for incubation

SERIES	EXPERIMENT	PEN 1 Ration fed to hen	PEN 2 Ration fed to hen
1 (1947)	1	Low-protein	High-protein-casein
	2	Low-protein	High-protein-casein
	3	High-protein-soybean	Low-protein, plus dried cow manure
2 (1948)	4	Low-protein	High-protein-casein
	5	Low-protein	High-protein-casein
	6	Low-protein	High-protein-casein
	7	Low-protein	High-protein-casein
	8	High-protein-soybean	Low-protein
	9	High-protein-soybean	Low-protein

Incidentally, the egg production of hens on the high-protein diets was higher than that of hens kept on low-protein feed. After a three-month period, some of the hens on low-protein rations stopped laying. This accounts for the small number of chicks from hens on this particular diet in the experiments in the 1948 series in which mash supplemented with dried cow manure or extract was fed.

The eggs laid by the hens in the two pens were gathered daily, marked for identification, and placed in a cooler at 55°F. Once each week the eggs were placed in forced-draft incubators that maintained a temperature of 100°F. and a relative humidity of 55%. At hatching time each chick was weighed and wing-banded.

Chicks hatched from eggs gathered in respective experiments were fed the chick mash (table 1) with or without sup-

plements. Chicks of hens fed low-protein rations served as controls in all instances. Chicks fed an identical ration, in any one experiment, whether in an experimental or control group, were caged together weekly (50 at a time) in the same compartment of an electrically heated battery brooder. Feed and water were supplied ad libitum. Individual chicks were weighed when 14 days and 28 days of age, but only the weights at 28 days were used for calculations.

The average gain of weight, shown in table 3, was calculated from the combined weights of all chicks of the same type reared in each experiment. In the statistical analysis (T value), comparison was made between the progenies of hens in pen 1 and pen 2.

Comparisons of experiments within the same pen in the same series furnished additional data.

RESULTS AND DISCUSSIONS

The series of experiments carried out in 1947 and again in 1948 showed that when the chicks were fed the same ration, consisting of mash only, those of hens fed high-protein rations containing casein gained more in weight than those of hens fed low-protein rations (table 3). In 1947 the average weight gains were, respectively, 240.8 gm and 196.9 gm, and in 1948 they were 203.5 gm and 179.0 gm. The differences were found to be highly significant. It should be emphasized again that all chicks in each experiment were caged together from hatching to their 28th day, and fed the same chick mash. The only difference observed between chicks with the two types of prenatal nutrition was that one gained weight faster than the other. The only difference in the food of their mothers was that the mothers in pen 1 received the low-protein diet in which the protein came mainly from corn, while those in pen 2 received the high-protein diet containing 20% casein.

The same conclusion was reached when the progeny of penmates in the 1948 series were compared. Chicks of hens fed high-protein rations containing casein gained an average of

TABLE 3

Average weight gain of chicks from eggs of hens fed various protein diets after feeding 28 days on mash alone and on mash with various supplements

YEAR OF SERIES AND RATION FED TO MOTHER HEN	PEN OF MOTHER HEN	RATION FED TO CHICKS							
		Mash only		Mash supplemented with					
				5% cow manure				0.2% liver fraction "L"	
				Dried		Extract			
		Number of chicks	Average weight gain	Number of chicks	Average weight gain	Number of chicks	Average weight gain	Number of chicks	Average weight gain
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
			gm		*gm*		*gm*		*gm*
1947 series									
Low protein	1	83	196.9	42	215.6				
High protein (casein)	2	109	240.8	39	191.6				
Difference		..	43.9	..	24.0				
T value			6.27 [1]		2.58 [2]				
1948 series									
Low protein	1	67	179.0	14	189.0	13	194.8	34	175.0
High protein (casein)	2	81	203.5	40	162.0	40	196.5	98	171.0
Difference		..	24.5	..	27.0	..	1.7	..	4.0
T value			3.93 [1]		3.57 [1]		0.2 [1]		0.85
1947 series									
Low protein, plus 5% dried cow manure [3]	2	33	180.3						
High protein (soybean) [4]	1	74	199.3						
Difference		..	19.0						
T value			1.88 [1]						
1948 series									
Low protein [3]	2	67	164.2	28	162.0				
High protein (soybean) [4]	1	122	154.0	68	164.0				
Difference		..	10.2	..	2.0				
T value			2.16 [2]		0.53				

[1] Statistically highly significant change in weight. Exceeds the 1% level according to Fisher's "T" table (Fisher, '36).
[2] Statistically significant change in weight. Exceeds the 5% level according to Fisher's "T" table (Fisher, '36).
[3] Ration was changed. Previously on high-protein (casein) ration.
[4] Ration was changed. Previously on low-protein ration.

203.5 gm, while those of hens fed low-protein rations gained only 164.2 gm. On the other hand, chicks of hens fed high-protein rations containing soybean meal showed less gain in weight than the control chicks. The differences are statistically significant. Results obtained with the progeny of pen-mates confirmed these findings. The chicks of hens fed high-protein rations containing soybean meal gained an average of only 154.0 gm and the chicks of hens fed low-protein rations 179.0 gm.

These results indicate that there was present in the diets fed a hitherto unrecognized growth factor. The carrier of this factor in the hens' diets would seem to have been casein as the most effective, corn as intermediate, and soybean meal as the least effective under our experimental conditions, since these were the only varying constituents in the rations.

Several tests were conducted in the course of these studies to establish a relationship between the growth factor found by us and the one which has been reported to be present in cow manure and in liver fraction "L." Bird et al. ('48) showed that the addition of 5% dried cow manure to their chick ration resulted in increased chick growth. However, in our studies, when 5% cow manure dried at 45°C. was added to the chick mash, chicks from hens fed the high-protein-casein ration grew more slowly and weighed less at 4 weeks than the controls. When comparison was made among the chicks of hens fed low-protein diets in the 1947 experiments, it was found that the chicks receiving mash supplemented with cow manure gained more weight than those receiving the mash only. In other words, the addition of dried cow manure to the chick ration: (1) caused a marked inhibition of growth in the high-protein-casein chicks; (2) had no effect on the growth of the high-protein-soybean chicks; and (3) caused a slight increase in the rate of growth of the low-protein chicks.

When chicks of hens fed low-protein rations containing dried cow manure were fed on mash only, their weight gains were not accelerated. This result indicated that the cow manure growth factor was not transmitted to progeny under the con-

ditions of this experiment. Under somewhat different experimental conditions, Rubin and Bird ('46) concluded that the factor was transmitted.

A sample of cow manure extract furnished by Dr. Bird (the preparation of which has been described by Rubin and Bird, '46) and added to the chick mash caused only a slight inhibition in the growth of chicks of hens fed high-casein rations. Seemingly, the inhibitory factor in this preparation had been mostly removed; the average gain of these chicks in 28 days amounted to 196.5 gm. In comparison, the chicks of hens fed high-casein rations, when given mash with 5% dried cow manure, gained only 162 gm; on mash alone the chicks gained 203.5 gm. The addition of cow manure extract to the mash produced accelerated weight gains in chicks of hens fed low-protein rations.

These results indicate the presence of a growth-inhibiting and a growth-promoting factor in dried cow manure, yet they do not explain why growth was inhibited in the chicks of hens fed high-protein rations containing casein but not in those of hens fed low-protein rations.

Wilson's liver fraction "L" is also supposed to contain a growth factor. However, when it was fed under our experimental arrangements no growth-stimulating effect was observed; the average gain in body weight both of chicks of hens fed low-protein rations and those of hens fed high-protein rations containing casein was practically the same. However, if we compare the average gain of high-protein-casein chicks in column 10, pen 2 (table 3), with that of high-protein casein chicks in column 4, same pen, the inhibitory effect of the liver fraction "L" and not the growth-promoting effect is found to be the prevailing one. Under our experimental conditions the inhibitory effect on the low-protein chicks was slight.

It is doubtful whether the agent causing accelerated growth in the high-protein-casein chicks is identical with those growth factors already reported in the literature in connection with casein feeding, such as factor X (Cary et al., '46), strepogenin (Woolley, '46), and factor S (Scott et al., '47). First, the

growth effect we describe was transmitted from the casein-fed hen through her egg to the chick; none of the chicks received casein in its diet. Secondly, we found no accelerated growth of chicks from hens fed a high-protein-soybean diet; on the contrary, with such a diet the rate of growth was actually less than the growth rate of the low-protein chicks (column 4). Strepogenin and factor S have been shown to be present in soybean meal (Woolley, '46; Scott et al., '47). Additional heating of commercially obtained soybean meal was necessary before the activity of factor X could be noted (Cary et al., '46). However, the results we obtained with liver fraction "L" definitely eliminate factor X as being responsible for the growth effect.

Our experimental arrangements were such as to exclude the possibility that any known deficiency was a factor in the results we obtained. The chicks whose growth served as a criterion were reared together and fed a diet adequate in all nutrient factors known to be essential for chick development. The explanation of the better growth of the high-protein-casein chicks, observed during the 28-day period, in comparison to the low-protein chicks must reside in the differences in the dietary protein fed to the mothers of the chicks. We want to make it clear that, in our opinion, the transmittal of the growth factor we observed is not a result of inheritance as such, but strictly one of nutrition. We believe that the growth factor is incorporated in the hen egg, which serves as a food supply for the developing embryo.

SUMMARY

A group of hens in two separate pens were fed, alternately, low-protein and high-protein rations. High-protein rations were obtained by adding soybean meal and casein, respectively, to the basal diet.

The eggs produced were incubated and the chicks (reared together) were fed a chick mash. Twenty-eight days after hatching their weight gains were recorded.

It was found that hens fed a ration containing 20% casein transmitted a growth factor through their eggs to the chicks.

With soybean proteins in the ration the same effect was not observed.

The addition of dried cow manure or Wilson's liver fraction "L" to the chick mash inhibited the growth of chicks from hens fed the high-protein-casein diet.

The addition of cow manure dried at 45°C. to the chick mash slightly accelerated the growth of chicks from hens fed a low-protein ration, but failed to affect the growth of chicks from hens fed a high-protein-soybean diet.

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NUTRIENT ANALYSES OF UNITED STATES FOOD SUPPLIES

ANALYSES OF COMPOSITED SAMPLES OF OUR NATIONAL FOOD SUPPLIES AND OF THE WHITE FLOUR AND NON-WHITE FLOUR COMPONENTS THEREOF IN TERMS OF VARIOUS NUTRIENTS

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Advances in knowledge of the composition of available food supplies and potential food resources, in relation to man's food requirements in terms of specific nutrients, command ever increasing interest.

With very few exceptions, previous publications on assessments of specific nutrients provided in national food supplies have been based on computations derived by use of tables of "average or representative" composition of the individual food items involved. Obviously, both this method and the method of direct analysis possess certain advantages and disadvantages. Considerable interest, therefore, attaches to comparative results obtained by the two methods.

One of the more recent and most extensive assessments of United States food supplies includes computed estimates of the apparent per capita consumption of major foods in terms of their retail weights and computations of the specific nutrients carried therein (Bureau of Human Nutrition and Home Economics, '46). This report includes a table of computed val-

ues for 11 nutrients "available for consumption" per capita per day for the civilian population of this country for the years 1909–1945. Another recent report deals with computations of the nutrient composition of national food supplies (in terms of 11 nutrients) for the civilian populations, per capita per day, for the United States, Canada and the United Kingdom for the pre-war years 1943 and 1944 (Combined Food Board, '44). An attempt has also been directed toward a world food survey by the Food and Agriculture Organization of the United Nations ('46), which culminated in a report of computations of the caloric values and protein content of the pre-war food supplies of 70 countries.

Each of the three publications just cited refers to the disappearance of nutrients presumably supplied by the edible portions of the food involved. These computations include average deductions for inedible refuse but make no allowances for other ways that food or food-nutrients can be lost or wasted in homes and public eating establishments.

Seemingly the only published accounts of attempts to assess the various nutritive values of a national dietary by means of direct analyses, and with due allowances made for nutrient losses in cooking, are those of Williams and his co-workers. The two reports of this work (Lane, Johnson and Williams, '42; Cheldelin and Williams, '43) are concerned chiefly with thiamine and with the riboflavin, nicotinic acid and pantothenic acid contents, respectively, of the "average American diet" after all foods have been prepared for serving. On this basis, the average American diet was reported to carry about 0.8 mg of thiamine, 1.4 mg of riboflavin, 11 mg of nicotinic acid and 4.9 mg of pantothenic acid, per 2,500 cal., provided none of the white flour or bread component was enriched. Substantial increases in the thiamine, riboflavin, nicotinic acid and iron contents of the diet would perforce accrue by virtue of enrichment of the white flour component or of breadstuffs made with white flour. An estimate of the increments contributed in terms of the three vitamins in the enrichment ingredients based upon earlier enrichment standards (Federal Register,

'41) was computed by Williams and his associates, but each of these would require recomputation to be in accord with the upward revision of the standards for these nutrients in enriched flour (Federal Register, '43) promulgated subsequent to publication of the Williams et al. reports.

The present investigation was designed primarily to determine by direct analyses of composited sample food mixtures the various nutrient contents of our national dietary with and without such nutrient contributions as are potentially available in the form of enriched flour-converted products, all food items in the mixtures having been prepared for serving. Some further work was included to provide information on nutrient losses occasioned by conversion of enriched flour to enriched flour-converted products, and on those losses occasioned by the cooking of such other food items as are not generally eaten in the raw state.

Analyses of national food supplies, especially after the preparation of the food for serving, quite naturally provoke an inquiry as to the nutritional evaluation of the national dietary. Any resolution of such an inquiry presupposes the existence of some appropriate standard of reference for comparison, a matter which was not a subject of study in the observations reported here. However, the dietary allowances for 10 nutrients as recommended by the Food and Nutrition Board ('43, '45, '48) could, within the limits of a rough approximation, be computed in terms of daily per capita allowances by taking into account the general distribution of the population as regards age, sex and activity. These data, combined with knowledge of the nutritional contributions of national food supplies after all items therein have been prepared for serving, should at least provide an indication of whether a national dietary is potentially or inherently capable of meeting the recommended dietary allowances for such nutrients as were covered by the analyses. For the purposes of such an appraisal the authors of the present article have included weighted averages for the recommended dietary allowances in terms of specific nutrients for the estimated composition of the civilian population of the

United States as of July 1, 1944,[1] in accord with the latest revision of these allowances (Food and Nutrition Board, '48).

EXPERIMENTAL

Proportions of various food items in a mixture considered representative of our national dietary

In table 1 are listed the individual food items, together with the quantities of each, included in 3,000-cal. allotments of the complete food mixture analyzed as a representative sample of the edible portions of United States food supplies in recent years. In planning for the kinds and proportions of various food items to be used in the sample food mixtures, full use was made of the most recent annual publication of Agricultural Statistics (U. S. Department of Agriculture, '47), of a recent publication of the Bureau of Human Nutrition and Home Economics ('46), and of a paper on the average American diet by Lane, Johnson and Williams ('42). For the conversion of purchased weights of food into edible portions of food, considerable recourse was made to the tabulated data of Chatfield and Adams ('40).

All of the analytical data are given in terms of the quantities of the respective nutrients in 3,000-cal. allotments of the complete food mixture or of some designated component part thereof, whichever is specifically indicated. The 3,000-cal. base was selected by the authors because this allotment would appear to be very close to the apparent per capita consumption rate per day in this country for the combination of all foods as prepared for serving at the nation's tables.

Space does not permit our giving a detailed account of the basis for selecting the proportions of the approximately 75 different food items included in the complete food mixtures

[1] The composition of the civilian population of the United States corresponding to the various age-sex-activity classifications designated in the Food and Nutrition Board's listing of recommended dietary allowances was provided by Dr. Margaret Haywood, Bureau of Agricultural Economics, U. S. Department of Agriculture, through Dr. Frank L. Gunderson, during the latter's period of service as Secretary of the Food and Nutrition Board, National Research Council.

analyzed. In lieu of this, the reader's attention is called to the data in column 4 of table 1, where the authors have set forth the annual rates of apparent consumption of the foods included ("as purchased" basis), which would correspond to the 3,000-cal. allotment in terms of edible portions. A further judgment of the rationale of compositing the edible portions of the foods as indicated in table 1 can be made by comparison of the data in column 4 with data on apparent per capita consumption rates released in recent years by two Bureaus of the United States Department of Agriculture (see columns 5 and 6 of table 1).

One detail with regard to fats, other than fat-spreads, should perhaps be mentioned here, since in the present report the treatment of this item marks a distinct departure from that with regard to any other foodstuffs listed in table 1. Much of the cooking fat and fat rendered from fat cuts of meat, although purchased as food, does not customarily appear on American tables. This fact was well borne out in domestic fat-salvage programs during the World War II period and in surveys of food wastage made during 1917–19 as reported by Pearl ('20). For this reason, the authors have deleted from the food mixtures analyzed about 10 lb. of cooking fat per capita per year, as a conservative allowance for the amount of fat purchases which would not appear as food prepared for serving.

Preparation of sample food mixtures

The individual food items to be combined in the mixture representative of the national food supply were purchased in the local retail markets of Minneapolis in early July, 1948. The kinds and relative quantities of the various food items included in the complete food mixture are shown in table 1, columns 1 and 2 respectively. Multiples of the quantities of foods shown in column 2 were composited for analyses in terms of the constituents indicated in the following section on Methods of Analyses.

TABLE 1

Kinds and relative proportions of individual food items included in mixtures representative of United States food supplies

(Authors' data are based on 3,000-cal. allotments of the complete mixture and annual consumption rates corresponding thereto; comparative data are based on apparent annual per capita consumption rates)

FOOD ITEM	IN 3,000-CAL. ALLOTMENTS E.P. BASIS [1]	METHOD OF FOOD PREPARATION [2]	CORRESPONDING ANNUAL CONSUMPTION RATE, A.P. BASIS	ANNUAL APPARENT PER CAPITA (CIVILIAN) CONSUMPTION RANGE 1940–45 (Agricultural statistics) [3]	ANNUAL APPARENT PER CAPITA (CIVILIAN) CONSUMPTION RANGE 1940–45 (Estimated retail weights) [4]
	gm.		*lb.*	*lb.*	*lb.*
Grain products					
White flour, enriched	199.0	Made into bread and biscuits	160	147.4–164.2	
Whole wheat (100%) bread	7.8	.....	4 [5]		
Rye bread, commercial	5.2	.....	2 [5]	2.2–3.1	
Cornmeal, whole grain	14.3	Cooked in pressure saucepan	12	19.0–21.7	
Rice, white	6.5	Cooked in pressure saucepan	5	4.7–5.9	
Oatmeal, quick cooking	5.2	Cooked in pressure saucepan	4	3.2–4.8	
Prepared breakfast cereals	7.8	.....	6	5.7–6.3 [6]	
Total grain products	245.8		193	187.1–202.9 [6] (Excludes barley)	192–208
Dairy products (excl. butter)					
Milk, whole fresh	360.0	.....	290		
Milk, evaporated	26.0	.....	46 [7]		
Milk, dry, defatted	10.2	.....	98 [7]		
Cheese, commercial	9.3	.....	75 [7]		
Total dairy products (excl. butter)	405.5		509 [7]	466.1–567.2 [7,8]	.....
Total fluid milk equivalent			237 qt.	.. ..	215–257 qt.
Eggs, total weight	38.1	Soft cooked	37	.....	.....
number			316 eggs	311–397 eggs	302–374 eggs
Flesh foods					
Beef, fresh	15.6	Broiled	59 [9]	49.6–61.3 [9]	
Beef, fresh	35.2	Cooked in pressure saucepan			
Pork, fresh	13.1	Broiled	62 [9]	62.7–80.1 [9]	
Pork, fresh	14.3	Cooked in pressure saucepan			
Pork, cured (other than bacon)	13.1	Broiled	7 [9]	6.3–7.2 [9]	
Lamb, fresh	5.4	Broiled	8 [9]	7.4–12.3 [9]	
Veal, fresh	5.3	Fried	19 [9]	22.2–33.9 [9]	
Poultry, fresh	14.3	Cooked in pressure saucepan	27 [9]	.. ..	
Fish, fresh	18.5	Broiled			
Fish, canned	5.4	None, juice discarded			
Total flesh foods, incl. fish	140.2		182 [9]		148–160
Total flesh foods, excl. fish			155 [9]	163.9–182.8 [6,9]	
Potatoes and sweet potatoes					
Potatoes, white	136.8	Cooked in pressure saucepan, liquid discarded	131	125–133	
Sweet potatoes	17.2	Cooked in pressure saucepan	16	19.3–21.6	
Total white and sweet potatoes	154.0		147	145.9–154.6 [6]	137–146
Citrus fruits and tomatoes					
Grapefruit	12.4	None	43	56.3–67.8	
Oranges	24.8	None			
Tomatoes	21.8	None	37	34.6–36.9 [6]	
Tomatoes, canned	23.4	Heated			
Total citrus fruits and tomatoes	82.4		80	90.9–102.7 [6]	95–119
Other vegetables and fruits					
Asparagus, canned	5.2	Heated			
Beans, baked with pork, canned	9.1	Heated			
Beans, lima, canned	3.5	Heated			

Oranges	21.8	None	37	34.6–36.9 [6]	
Tomatoes	23.4	Heated			
Tomatoes, canned					
Total citrus fruits and tomatoes	82.4		80	90.9–102.7 [6]	95–119
Other vegetables and fruits					
Asparagus, canned	5.2	Heated			
Beans, baked with pork, canned	9.1	Heated			
Beans, lima, canned	2.5	Heated			
Beans, green snap	13.0	Cooked in pressure saucepan			
Beets	2.9	Cooked in pressure saucepan, liquid discarded			
Cabbage	10.6	Cooked in pressure saucepan, liquid discarded			
Cabbage	6.4	None			
Carrots	5.7	Cooked in pressure saucepan			
Carrots	1.8	None			
Cauliflower	5.3	Cooked in pressure saucepan, liquid discarded			
Celery	2.5	None			
Corn, whole kernel, canned	16.9	Heated			
Lettuce, head	13.6	None			
Okra, canned	4.4	Heated			
Onions, bulb	26.3	Cooked in pressure saucepan			
Peas, canned	16.9	Heated			
Peas, and beans, dried	9.1	Cooked in pressure saucepan			
Pumpkin, canned	9.1	Heated			
Radishes	2.5	None			
Sauerkraut, canned	3.9	Heated			
Spinach	6.5	Cooked in pressure saucepan			
Turnips	4.7	Cooked in pressure saucepan, liquid discarded			
Apples	18.5	Cooked in open saucepan			
Apples	30.0	None			
Apricots, canned	2.5	None			
Bananas	21.2	None			
Grapes, Malagas	6.0	None			
Muskmelon	4.8	None			
Peaches	6.0	None			
Pears	14.3	None			
Pineapple	6.5	None			
Prunes, dried	5.2	Cooked in open saucepan			
Raisins, dried	2.5	None			
Raspberries, red	6.5	None			
Watermelon	11.5	None			
Total other vegetables and fruits	314.4		322	330–395 [6]	318–370
Fats					
Fat (including fat spreads)	58.0	Part used in bread and biscuits	47	41.6–50.4 [6]	
Fat cuts, including bacon	9.2	Fried (fat used in cooking)	7	.. ..	
Total fat and fat cuts	67.2		54	.. ..	60–71
Sugar, or sugar and syrup, total	134.3	Part used in cooking	108	89.4–116.5 [6]	91–117
Miscellaneous					
Peanuts, roasted	1.9	None	2.0		
Chocolate	1.3	None	1.0		
Yeast, moist baker's	1.7				
Grand total	1586				

[1] Multiples of quantities shown in this column were used for compositing food mixtures 1 and 2, and mixture 3 except for omission of the enriched flour component in the white bread and biscuits.
[2] All cooking liquors, juices, and rendered fats included unless otherwise stated in this column.
[3] Data in this column taken directly from or computed from figures released in "Agricultural Statistics" (U. S. Dept. of Agri., '47).
[4] Data taken from "Nutritive value of the per capita food supply, 1909–45," Bureau of Human Nutrition and Home Economics ('46).
[5] This figure refers to average weight of flour in the type of bread indicated.
[6] Range of totals during 5-year period.
[7] Weight of fluid whole milk equivalent to product indicated.
[8] Computed by subtraction of fluid milk equivalent of butter from total fluid milk equivalent of dairy products.
[9] Dressed weight basis.

All non-perishable food items included in the food mixtures were assembled one to two days preceding laboratory preparation of the mixtures. All perishable items were delivered to the laboratory in the late afternoon of the day immediately preceding preparation of the mixtures, and were stored overnight under refrigeration (4.5°C.). Cooking, according to the methods indicated in column 3 of table 1, and other customary preparations of the various purchased food items, were begun early the following day according to a carefully prearranged schedule of timing, so that all items would be ready for mixing and blending at approximately the same time. A large share of the vegetable cookery was done in pressure saucepans, which further facilitated completing the preparation of these foods on schedule. As is indicated in table 1, expressed juices as well as cooking liquids were included in the sample mixtures except for cooking liquors associated with a few strongly flavored vegetables.

The blending of the individual food samples was accomplished by subjecting the specified kinds and quantities of food items to trituration in the glass receptacle of a motor-driven food blender. Except for such analyses as were initiated immediately after conclusion of the blending operation, analyses were made on aliquots of the mixtures which had been quick-frozen immediately after the blending of the food mixtures, and stored at a temperature of — 29°C.

The following food mixtures were assembled for analysis from single lots of purchased food items:

Mixture 1. Complete food mixture composed of the individual food items of the kinds and in the quantities specified in table 1 and with cooking procedures confined to the enriched flour component, 50% of this flour having been made into yeast-raised bread and 50% into baking powder biscuits.

Mixture 2. Complete food mixture, the same as mixture 1 except that all food items were prepared for serving prior to the blending of the individual food items.

Mixture 3. Identical to mixture 2 except that the flour, leavening agents, salt, yeast food and malted grain in all the enriched flour-converted products were excluded.

In addition to analyses of the mixtures described above, separate analyses were made on an aliquot of the enriched flour sample which was purchased along with other items at a local market and employed in making the bread and biscuits included in mixtures 1 and 2.

Since one of the objectives of this study was to determine the *potential* contributions of enriched flour to the national food supply, rather than those of some arbitrarily selected or assumed proportion of enriched and unenriched flour, the entire white flour-converted components in mixtures 1 and 2 were made with enriched flour. The selection of bread [2] and biscuits [3] as carriers of the nutritive contributions of the enriched flour component in mixtures 1 and 2 is, of course, a decided simplification of the many uses to which white flour is put in our national dietary. However, the authors considered that this simplification in procedure would yield about average nutritive values for such heat-labile nutrients as would be involved in the wide assortment of uses to which white flour is currently put in this country.

The total quantities of cooking fats and of sugar (sucrose) incorporated in *each* of the three food mixtures were the same whether portions of these two items did, or did not, appear as ingredients of bread and biscuits. The remainder of the non-flour ingredients used in making the bread and biscuits (yeast, malted grain, yeast-food, salt and the phosphate type of baking powder) were included only in mixtures 1 and 2, wherein the flour components were represented as enriched flour-converted products. This procedure was adopted after due consideration of the distortion of analytical results to be anticipated by introducing so questionable a procedure as

[2] Bread formula (all amounts in grams): Enriched flour 100, water 64, hydrogenated fat 3.5, sugar 5, salt 2, yeast 2, malted grain 1, yeast food 0.33.

[3] Biscuit formula (all amounts in grams): Enriched flour 100, hydrogenated fat 15.38, baking powder 14.8, sugar 2.50, salt 2, water 85.

having live yeast and unspent baking powder intimately associated with the constituents of mixture 1. Neither of these substances is normally associated in human dietaries with other than flour-conversion products.

All cooking procedures applied to either vegetables or meats were conducted with the objective of retaining maximum nutritive values. The reader will recognize this as a departure of indeterminate magnitude from average cooking practices in kitchens throughout the nation. It was decided that any arbitrarily selected variations with regard to conservation of nutrients would have introduced a situation very difficult, if not impossible, to evaluate, and would have been entirely inconsistent with the over-all objective of investigating the potential maximum of nutritive values in national food supplies.

The beef, pork and lamb samples included in the mixtures were selected in each case as representing, in our judgment, medium fatness and good quality. In so far as it was possible to do so, individual fruit and vegetable items were included in the food mixtures in the form or forms (fresh, canned, dried) most widely purchased by consumers. A few of the less common fruits and vegetables, grown and consumed in this country but not included because of their inaccessibility, were replaced by items having similar usages. This statement is made in explanation of the fact that the food items incorporated in the complete food mixtures (table 1) show omissions of some of the less common or regionally restricted fruits and vegetables. It was obviously impossible to select absolutely *identical* samples from retail purchase lots of such structurally inhomogenous items as meats and vegetables for inclusion in the raw and prepared food mixtures, respectively. However, maximum care was taken to secure as nearly matched samples as visual perception permitted.

Methods of analysis

The methods of analysis used in determining the quantities of various constituents in food mixtures 1, 2 and 3 and in the enriched flour are indicated in table 2.

The analyses for thiamine, riboflavin and nicotinic acid were initiated immediately following the blending of the food mixtures. The remainder of the analyses were made on aliquots of the same food mixtures which had been quickly frozen and stored in the frozen state at a temperature of — 29°C.

TABLE 2

Analytical methods employed in the determination of various constituents in enriched flour and in food mixtures 1, 2 and 3

CONSTITUENT	METHOD
Nitrogen	Kjeldahl method as outlined by Assn. Offic. Agri. Chem. ('45), p. 26.
Protein quality	Nitrogen balance (using young rats 50–60 gm in weight) in terms of whole egg protein replacement value; Sumner, Pierce and Murlin ('38).
Ash	Assn. Offic. Agri. Chem. ('45), p. 238.
Crude fiber	Assn. Offic. Agri. Chem. ('45), p. 409.
Fat	Acid hydrolysis as outlined by Assn. Offic. Agri. Chem. ('45), p. 240.
Calcium	Assn. Offic. Agri. Chem. ('45), p. 415.
Phosphorus	Ammonium vanado-molybdate method as adapted to cereal products by the General Mills analytical research staff, based on methods of Murray and Ashley ('38) and Willard and Center ('41).
Iron	α, α-dipyridyl method, Am. Assn. Cer. Chem. ('47), p. 47.
Potassium	Volumetric chloroplatinate method of Gerritz ('42); also flame photometer method conducted for us by the Laboratory of Vitamin Technology, Chicago, Ill.
Thiamine	Am. Assn. Cer. Chem. ('47), p. 114.
Riboflavin	Am. Assn. Cer. Chem. ('47), p. 121.
Nicotinic acid	Microbiological method of Andrews, Boyd and Gortner ('42).
Pyridoxine	Yeast growth method of Atkin et al. ('43), conducted for us by the Laboratory of Vitamin Technology, Chicago, Ill.
Pantothenic acid	Microbiological method of Skeggs and Wright ('44), conducted for us by the Laboratory of Vitamin Technology, Chicago, Ill.; same method modified to include clarase digestion, conducted for us by Food Research Laboratories, Inc., Long Island City, N. Y.
Moisture	Assn. Offic. Agri. Chem. ('45), p. 382, modified to include overnight drying.
Vitamin E	Biological assay following the principle of Palmer's ('37) method.

In the cases of moisture, ash, nitrogen, fat, crude fiber, calcium, phosphorus, iron and potassium, two or more entirely separate analyses were made for each constituent, with each analysis including determinations made in either duplicate or triplicate. The values shown in this report represent simple averages of the two or more sets of analyses made at different times.

The thiamine analyses were made in triplicate under the supervision of two analytical chemists working independently of one another. The values for thiamine represent simple averages of these three separate analyses.

The analyses for pyridoxine and pantothenic acid and for potassium by the flame photometer method were, as is indicated in table 2, conducted for us in outside laboratories.

Appreciable amounts of water were required to be added to the food mixtures during the blending process to facilitate mixing. Moisture determinations made on these diluted mixtures, therefore, served to provide the means for computing the contributions of the various constituents to the total weights of the mixtures, but no attempt was made to make direct determinations of the moisture contents of the undiluted food mixtures themselves.

RESULTS

In table 3 are given the results of the analysis of samples of the three composited food mixtures and the enriched flour described earlier. The data shown in columns 2 to 5 inclusive refer to the quantities of respective constituents found in approximately 3,000-cal. allotments of the complete food mixtures (or indicated component parts thereof) representative of our national food supplies in recent years.

A 3,000-cal. allotment of United States food supplies corresponds roughly to about 1.6 kg of fresh foods (E. P. basis) and to approximately 0.7 kg of edible dry food-substance. Thus, in spite of the lack of direct determination of the moisture contents of the undiluted food mixtures, it can nevertheless be stated that our over-all national food supplies are comprised,

very roughly, of 40% and 60% of dry food substance and moisture, respectively.

TABLE 3

Analytical data showing nutrient composition of food mixtures 1, 2 and 3 (see text) and of uncooked enriched flour ingredient included in mixtures 1 and 2 in the form of bread and biscuits. (Computed data in column 6 included for comparative purposes)

CONSTITUENT ANALYZED	COMPLETE NATIONAL DIETARY WITH ALL ITEMS EXCEPT ENRICHED FLOUR UNCOOKED (Mixture 1)	MIXTURES PREPARED FOR SERVING		ENRICHED FLOUR COMPONENT (199 gm)	COMPUTED RECOMMENDED DIETARY ALLOWANCES PER CAPITA PER DAY [1] (1948 revision basis)
		Complete national dietary (Mixture 2)	Mixture 2 with enriched flour ingredient omitted (Mixture 3)		
Protein (N × 6.25), gm	91.	93.	67.	..	64.
Protein (N × 5.7), gm	..	..	..	21.	..
Protein quality (% egg protein replacement)	81.	84.	88.	65.	..
Ash, gm	23.1	23.9	11.8	0.8	..
Fiber, gm	8.	9.	7.	0.4	..
Fat (acid hydrolysis), gm	94.	99.	92.	2.4	..
Carbohydrate (by difference), gm	447.	447.	280.	151.	..
Calories	3000.	3050.	2215.	708.	2610.
Calcium, gm	1.03	1.04	0.78	0.03	1.1
Phosphorus, gm	2.95	3.06	1.12	0.18	..
Iron, mg	17.7	20.4	9.8	7.5	11.7
Potassium (chem. method), gm	3.0	3.0	2.7	0.2	..
Potassium (flame photom.), gm	3.2	3.0	2.9	0.1	..
Thiamine, mg	2.03	1.78	1.09	0.96	1.2
Riboflavin, mg	2.13	2.14	1.56	0.48	1.6
Nicotinic acid, mg	20.7	20.2	13.2	8.2	12.6
Pyridoxine, mg	1.7	1.5	1.4	0.1	..
Pantothenic acid, mg	8.1	8.0	5.1	1.1	..
Dry weight of food mixture, gm	663.	672.	458.	175.	..

[1] For source and derivation of these figures, see text, pages 497 and 498.

According to the analytical data set forth in table 3, the civilian population of the United States derive about 12% of the total caloric value of their food from protein, about 28% from fat and about 60% from carbohydrates.

The protein in composited samples of our over-all national food supply, all items in the mixture having been prepared for serving, was observed to have a whole egg protein replacement value of 84%, while the protein in the same diet minus the entire white flour component showed a whole egg protein replacement value of 88%. The proteins of the white flour, the white bread and the biscuits showed no significant differences in biological quality, each exhibiting a whole egg protein replacement value of approximately 65%. Thus white flour proteins, providing about 25% of the total protein in our national dietary, alter the over-all protein quality of the dietary in only small degree from that found in the composited non-flour component.

The calcium and phosphorus contents of food mixture 1, as well as of food mixture 2, were approximately 1.0 gm and 3.0 gm, respectively. The calcium and phosphorus contents of our national dietary, exclusive of baking powders, yeast foods and all leavening agents carried by phosphated and self-rising flours, would be of the order of 0.80 gm and 1.3 gm, respectively, per 3,000 cal. It is clear, however, that various materials other than yeast which are commonly used as leavening agents contribute significantly to the available dietary supplies of calcium and phosphorus of the American people. These also include phosphated and self-rising types of white flour, widely consumed in southern and southwestern regions of the United States, neither of which was included in the samples of the national dietary analyzed by us because of the apparent lack of reliable records of consumption rates on a nationwide scale.

The iron content of United States food supplies amounted to about 18 to 20 mg per a 3,000-cal. allotment of food, which quantities include iron derived from tap water used in cooking and from contamination by cooking utensils.

Except in the case of thiamine our observations have not indicated any *necessity* for the incurrence of B vitamin losses of much over-all practical significance as a result of simple cooking procedures. The thiamine value of a 3,000-cal. allotment of United States food supplies prior to preparation of the individual food items for serving was observed to be about 2.3 mg (2.03–[1.78–1.09] + 0.96); the same quantity of food after preparation for serving was observed to carry about 1.8 mg of thiamine, or about 20 to 25% less than was present in the total raw foods. These data refer to the complete food mixtures when the entire white flour component is enriched and when all cooking procedures are conducted with strict attention to conservation of the maximum thiamine values. The unavoidable losses in thiamine occasioned by preparation of the foods for serving were sustained in approximately equal proportions by the enriched flour and non-flour components.

The white flour component, assuming all of it to be enriched, would provide (in addition to about one-fourth of the total protein, around one-third of the carbohydrate and from 2 to 3% of the fat) approximately the following percentages of the other nutrients in United States food supplies after careful preparation of the foods for serving: 40 of thiamine, 25 of riboflavin, 35 of nicotinic acid and at least 45 of iron. The white flour component also provides appreciable amounts of calcium, phosphorus, potassium, pyridoxine and pantothenic acid, although the practical importance of these would appear to be minor except in dietaries where flour is consumed in more-than-average quantities.

The results of attempts to assess the vitamin E values of the mixtures representing our national food supply before and after preparation were not entirely satisfactory because: (a) the quantities of the food mixtures required to be fed over the 10-day supplementary feeding period greatly lowered, and at some levels of feeding entirely displaced, consumption of the vitamin E-free ration; and (b) it was quite obvious that major destruction of the vitamin E activity in food mixture 1 had taken place within the 15-day interval between preparation

of the mixtures and completion of the 10-day feeding period. The results, in comparison with those found with rats consuming the vitamin E-free ration supplemented with different levels of alpha-tocopherol, indicated a potency equivalent to about 18 mg of alpha-tocopherol per a 3,000-cal. allotment of our national food supplies after preparation for serving, and only about one-third of this amount in the same quantity of food mixture 1. Obviously the vitamin E determination made on food mixture 1 is not valid. To what extent the destruction of vitamin E or the displacement of the vitamin E-free ration during the 10-day feeding period may have affected the results obtained for mixture 2 cannot be assessed, but it is obvious that major destruction of the vitamin E activity had taken place in food mixture 1 despite low temperature storage (— 29°C.) of the material prior to feeding. In view of the obvious rapid destruction of vitamin E activity in at least one of the food mixtures, this work was not followed up with attempts to reassess the vitamin E activity, using fat-solvent extracts of the mixtures.

Difficulties analogous to, or even more serious than, those encountered with vitamin E could reasonably be expected in attempts to determine vitamin A and ascorbic acid values in composited and blended food mixtures, and for this reason these latter two vitamins were left entirely out of consideration in this study.

DISCUSSION

Since all of the analytical data presented in table 3 for enriched flour and for composited food mixtures 1, 2 and 3 represent observations for which samplings of the same lots of each food item concerned were employed, these data would seem to invite, or at least lend themselves quite logically to, various types of computable interrelationships. In this connection, however, the authors wish to emphasize that the data shown for enriched flour and for the three food mixtures are primary sets of data derived in each case by separate analyses. The well-known concept of additive experimental error, therefore,

cannot be disregarded as a factor of potential importance in mathematical manipulations of these data.

On the basis of data presented here and elsewhere there should be no difficulty in making comparisons of the nutrient compositions of United States food supplies (prepared for serving) with and without benefit of enrichment of the white flour component. Obviously the substitution of unenriched flour for an equal weight of enriched flour would not alter the nutrient composition of our national dietary except as regards nutrients included as enrichment ingredients. Lane et al. ('42) and Cheldelin and Williams ('43) have already reported in earlier issues of this Journal the thiamine, riboflavin and nicotinic acid values of United States food supplies when the entire white flour component was unenriched. The data of these authors show a 3,000-cal. allotment of our national food supplies to contain 0.92 mg thiamine, 1.7 mg riboflavin and 13 mg nicotinic acid; which findings in the light of various types of information assembled in this laboratory, appear to be thoroughly representative, yet stand in marked contrast to the respective values for these three nutrients in our mixture 3 (see table 3), wherein the entire white flour component was enriched.

It will be noted from data presented in table 3 that, even after making due allowance for the difference in protein factors used in expressing the protein content of the enriched flour and non-flour components, the sum of the protein in these two fractions is slightly less than the protein content of either of the complete food mixtures. The shortage is presumably to be accounted for by sampling errors and by the relatively small protein contributions of yeast and malted grain, which were included only in complete food mixtures 1 and 2.

Any rational evaluation of the data presented in this report with the objective of arriving at an opinion or judgment of the nutritive adequacy of our over-all national food supplies must take into account the fact that our data make no allowances either for food wastage in homes and public eating establishments, other than for 10 lb. rendered or cooking fat

per capita per year, or for unnecessary losses in food nutrients occasioned by over-cooking or by holding foods at elevated temperatures after cooking. The only report of a large-scale investigation of food wastage known to the authors is that of Pearl ('20), who estimated that during the years 1917–19 at least 25% of the fat, probably as much as 20% of the carbohydrate, and about 5% of the protein in our national food supplies appeared in the nation's garbage.

In connection with Pearl's observations it might also be of interest to point out that there appears to be a decided trend toward over-consumption or wastage of food as the family income and the amount of income expended for food rise above family subsistence levels (Booher, '48). Moreover, according to the reports of several investigators (Briant et al., '46a, b; Goodhart, '43; Heller et al., '43; Nagel and Harris, '43; Sarett et al., '46; Streightoff et al., '46; Wertz and Weir, '44), the degree of retention of specific heat-labile nutrients in foods prepared and served in public eating places bespeaks an urgent need for improvement in cooking procedures in order to avoid unnecessary losses of thiamine, ascorbic acid, riboflavin and nicotinic acid values in foods. Possession of adequate food supplies alone will, obviously, not assure an adequate national dietary.

By combining the data shown in table 3 for enriched flour, mixture 1 and mixture 3, one can arrive at figures for the respective nutrients present in the composited food supplies in the raw or unprepared state. According to our observations, the *minimum* loss of thiamine to be anticipated as a result of careful preparation of our national food supplies is on the order of 20 to 25% of that in the original raw food, while losses of riboflavin, nicotinic acid, pyridoxine and pantothenic acid under the same conditions are of no practical significance. These observations, however, do not in the least conflict with the observations of others that maximum conservation of heat-labile nutrients falls far short of being common practice in our nation's kitchens.

As the result of data presented here, as well as the findings in various other publications herein referred to, it would seem appropriate to conclude that our over-all national dietary is inherently and *potentially* capable of providing the recommended dietary allowances (recommended intakes) of each of the 7 specific nutrients applicable to studies reported here. This potentiality, however, is contingent upon negligible food wastage, maximum conservation of food nutrients in the processes of food preparation, and use of a large proportion of the white flour component in the form of enriched flour (or an equivalent thereof).

SUMMARY

Analyses of food mixtures representative of United States food supplies in recent years in terms of proximate composition, protein quality, calcium, phosphorus, iron, potassium, thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid and vitamin E are herein reported.

The data presented provide the means for assessing the levels of these nutrients in the white flour and non-white flour components of our national dietary prior and subsequent to the application of simple cooking procedures to the individual foods included, the white flour component being represented, in accordance with the stated objectives of this investigation, as enriched flour.

It is demonstrated that the relatively large losses of thiamine and the lesser losses of other B vitamins reported by earlier investigators to have been encountered in various public or communal eating establishments represent, in large degree, unnecessary losses of food value.

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THE UTILIZATION OF CAROTENE FROM DIFFERENT SOURCES BY LAYING CHICKENS [1]

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FOUR FIGURES

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A number of investigators have shown that animals vary in their ability to convert carotenoid pigments into vitamin A. Differences have also been reported in the utilization by animals of carotenoids obtained from different sources. The above variations give rise to differences in the biological activity of carotenoids as regards their vitamin A potency. Callison and Orent-Keiles ('47) have shown that carotene derived from carrots is not utilized in the human body to the same extent as carotene obtained from other sources. Guggenheim ('44), working with rats, found that only from 33% to 67% of the carotene of plant origin is utilized as compared to the carotene in beef liver; the carotene from lettuce was found to be an exception, being utilized equally as well as that derived from liver. Hickman et al. ('44) and Harris et al. ('44) showed that natural mixed tocopherols enhanced the growth-promoting power of vitamin A and markedly influenced the vitamin A activity of carotene in the rat.

In the present investigation a study was made to compare the utilization by the chicken of vitamin A-active substances in the form of β-carotene, vitamin A esters from fish liver oil,

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and the naturally occurring carotenoids in fresh alfalfa and dehydrated alfalfa meal. The criteria for carotene utilization are based on liver storage, blood levels, and egg content of vitamin A and carotene. Frey et al. ('47) have presented data indicating that serum levels of vitamin A in cattle are a good index of vitamin A status in this animal. Within a wide range, liver storage was found to be approximately proportional to intake. The above observations have been confirmed by Hartzler ('48), using human subjects. Hoefer and Gallup ('47) compared the levels of vitamin A in the blood and livers of fattening lambs, the sources of carotene being alfalfa meal and carotene concentrates. Alfalfa meal was found to maintain a higher level of vitamin A in the blood than did carotene concentrates. Ross et al. ('48) found that Holstein heifers were capable of utilizing vitamin A alcohol and the natural esters of vitamin A more efficiently than carotene concentrates.

EXPERIMENTAL DESIGN

One hundred white Leghorn hens, approximately 9 months of age, were selected from a single flock on the basis of uniformity of size and condition. The birds were divided equally in 5 pens of the same size and shape. From February 4, 1948, to March 7, 1948 (33 days), they were fed the following basal diet, low in vitamin A activity: ground barley 37%, pulverized oats 25%, wheat gray shorts 20%, soybean oil meal 5%, meat and bone scrap 5%, pulverized limestone 3.5%, dried yeast 3%, steamed bone meal 1%, iodized salt 0.5%, D-activated animal sterol (2,000 AOAC U/gm) 25 gm %, and manganese sulfate 5 gm %. Blood samples were taken by wing puncture from 15 birds for vitamin A and carotene analysis at the time they were placed on the basal ration. On March 4 (30 days), two birds from each pen were sacrficed to determine liver stores and blood levels of vitamin A and carotene. Body reserves of the two constituents were found to have been sufficiently depleted to permit placing the remaining birds on the various supplements containing 2,000 I.U. of vitamin A per pound of ration consumed. The rations receiving their vitamin

A activity from carotene contained 1.20 mg of carotene per pound (assuming 0.60 μg of carotene to be equivalent to 1.0 I.U. of vitamin A). The rations receiving their vitamin A activity from fish liver oil contained vitamin A esters equivalent to 0.60 mg of vitamin A acetate per pound, as measured from a standard curve assuming 1.0 μg of vitamin A acetate to be equivalent to 3.33 I.U. of vitamin A. The following vitamin A active rations were fed: pen 1, basal diet; pen 2, basal diet + β-carotene in cottonseed oil; pen 3, basal diet + vitamin A esters from fish liver oil; pen 4, basal diet + fresh alfalfa; and pen 5, basal diet + dehydrated alfalfa meal. The experimental feeding period was terminated May 18, 1948 (72 days).

The supplements containing the vitamin A-active substances were fed Monday, Wednesday, and Friday of each week at 11 A.M. The calculated amount of vitamin A-active substance was weighed and mixed with a small amount of the basal ration and water to form a crumbly wet mash. The mash was placed in troughs and consumed within an hour after feeding. The fresh alfalfa used was cut in September, 1947, packed in cardboard containers, sealed, and quick frozen. When ready to be used the frozen alfalfa was ground in a food chopper, packed in tin cans, and again quick frozen. All supplements containing vitamin A activity were analyzed at frequent intervals for vitamin A and carotene content.

Blood samples were obtained by heart puncture from three birds in each pen on April 8. On April 26 one-half the birds from each pen were sacrificed to determine liver stores and blood levels of vitamin A and carotene. The remaining birds were sacrificed May 18. The whole liver was removed from each bird at the time it was killed, wrapped in wax paper, and quick frozen. At the beginning of the experiment three birds from each pen were selected and the yolk of each egg laid analyzed for vitamin A and carotene content. At the beginning of the experimental feeding period it was necessary to select several new birds to replace those which had ceased producing eggs. Trap nests were used for all birds and a record was

kept on egg production and ration consumption during the experimental period of 105 days.

The methods of analysis for vitamin A and carotene in blood and liver tissue were those described by Frey and Jensen ('46). The carotene content of egg yolk and alfalfa was determined by the "phasic" method given in an Association of Official Agricultural Chemists publication ('45). The vitamin A content of egg yolk was determined as in liver, using the petroleum ether fraction following separation of the carotene. Chromatographic methods for the separation of carotene in egg yolks have not proved satisfactory. For this reason it was thought advisable to use the "phasic" method for all carotene determinations other than liver and blood. Values for the carotene content of alfalfa, using chromatographic methods of analysis, compared favorably with the values found by the "phasic" method.

RESULTS AND DISCUSSION

The purpose of the preliminary feeding period of 33 days on the basal diet was to deplete the birds of their vitamin A and carotene reserves. The blood level at the beginning of the preliminary vitamin A depletion feeding period was 99.8 ± 59.3 μg of vitamin A and 211 ± 183 μg of carotene per 100 ml of serum. At the beginning of the experimental feeding period the blood level had dropped to 28.5 ± 9.7 μg of vitamin A and 33.1 ± 19.9 μg of carotene per 100 ml of serum. The latter values indicate fairly depleted reserves of the two blood constituents.

Figures 1 and 2 show the blood level and liver values of vitamin A and carotene during the experimental feeding period of 72 days. Complex variance analysis studies were made of vitamin A and carotene values for blood and liver samples obtained on April 26 and May 18. No significant difference between these dates was found within pens. However, highly significant differences between pens were found between these dates. Following are the the minimum differences required for significance (M. S. D.) between pens, for blood levels and liver

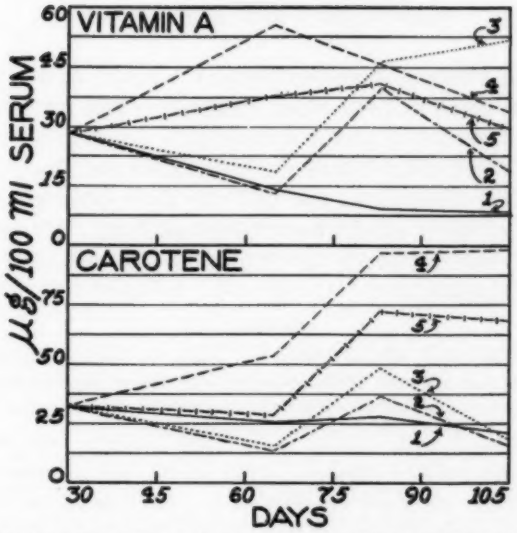

Fig. 1 Blood levels of vitamin A and carotene. 1. Basal ration. 2. Carotene supplement. 3. Vitamin A supplement. 4. Fresh alfalfa supplement. 5. Dehydrated alfalfa meal supplement.

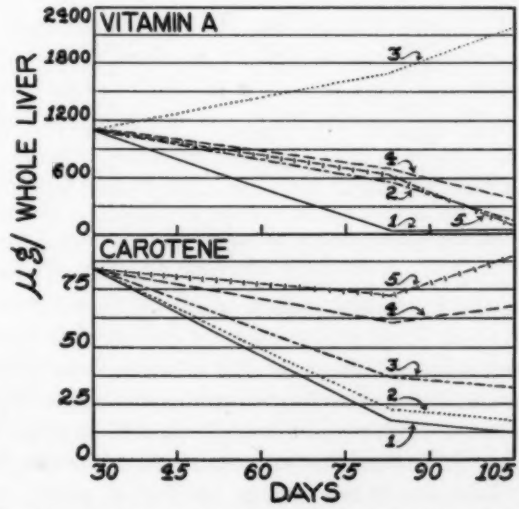

Fig. 2 Liver stores of vitamin A and carotene. 1. Basal ration. 2. Carotene supplement. 3. Vitamin A supplement. 4. Fresh alfalfa supplement. 5. Dehydrated alfalfa meal supplement.

reserves of vitamin A and carotene for April 26 and May 18: At the 0.01 level, 21.1 for blood vitamin A, 41.0 for blood carotene, 30.9 for liver vitamin A, and 31.5 for liver carotene; at the 0.05 level, 15.9 for blood vitamin A, 30.8 for blood carotene, 23.3 for liver vitamin A, and 23.7 for liver carotene. Table 1 gives the levels of significance, obtained from "t" values, for blood and liver reserves of vitamin A and carotene between pens, for May 18.

TABLE 1

Levels of significance, obtained from "t" values, for blood and liver reserves of vitamin A and carotene, between pens, after 72 days on the experimental diet (All birds were fed a preliminary vitamin A-depletion ration before being placed on the experimental diet)

PENS[1]	BLOOD		LIVER	
	Vitamin A	Carotene	Vitamin A	Carotene
1–2	0.001	0.40	0.10	0.01
1–3	0.01	0.90	0.01	0.05
1–4	0.001	0.01	0.01	0.001
1–5	0.001	0.10	0.05	0.001
2–3	0.02	0.80	0.01	0.001
2–4	0.02	0.05	0.10	0.001
2–5	0.02	0.10	0.50	0.02
3–4	0.20	0.05	0.02	0.001
3–5	0.10	0.10	0.01	0.01
4–5	0.50	0.50	0.05	0.30

[1] Pen 1: Basal diet only.
Pen 2: Basal diet + β-carotene in cottonseed oil.
Pen 3: Basal diet + vitamin A esters from fish liver oil.
Pen 4: Basal diet + fresh alfalfa.
Pen 5: Basal diet + dehydrated alfalfa meal.

The blood levels of carotene and vitamin A in the negative control pen, 1, did not decline significantly from March 4 to April 8, representing, respectively, 30 and 65 days on the basal ration. This indicates that minimum blood levels had been attained, which would not change until liver stores were further depleted. By April 26 (93 days) the liver reserves of vitamin A and carotene of the birds in pen 1 were practically

gone and blood levels were not significant, since the values were within the limits of error of the analytical methods used.

The birds in pen 3, receiving vitamin A, showed the same carotene values in the blood and liver as the birds in pen 1 receiving the basal ration. However, they showed the highest blood and liver vitamin A values, the liver values indicating that storage had occurred even though the blood level was only about one-half the value at the beginning of the experiment.

The birds in pen 2, receiving carotene, showed approximately the same blood carotene content as the control group in pen 1, even though liver reserves were somewhat higher. The blood and liver vitamin A values in pen 2 were significantly higher than in pen 1 after 53 days of the experimental feeding period, but were not significantly higher after 72 days. On the other hand, both fresh alfalfa and dehydrated alfalfa meal were more effective than β-carotene in maintaining body reserves of carotene. The carotene content of the blood and liver of the birds receiving alfalfa was higher than that of any of the birds in the other three pens at both 53 and 72 days. At 72 days the blood levels of vitamin A for the birds receiving alfalfa were higher than for the birds receiving carotene but lower than for the birds receiving vitamin A. The liver reserves of vitamin A of the birds receiving alfalfa closely approximated the values for the birds receiving carotene. The liver reserves of vitamin A for the birds receiving fresh alfalfa were significantly higher than those for the birds receiving dehydrated alfalfa meal, and significantly lower than those for the birds receiving vitamin A. None of the carotene-containing supplements approached vitamin A in effectiveness in restoring vitamin A reserves in the liver. It thus appears that β-carotene in cottonseed oil or the vitamin A-active substances in fresh alfalfa or dehydrated alfalfa meal are less effectively utilized as vitamin A than is vitamin A obtained from fish liver oil.

The tocopherols have been shown to be effective in aiding the utilization of carotene in the animal body (Hickman et al.,

'44; Harris et al., '44; Johnson and Baumann, '48). It is possible that the slight superiority of alfalfa over carotene, with respect to utilization as vitamin A, was due to the tocopherol content of the alfalfa. However, the tocopherol content of the basal ration was calculated to supply approximately two and one-half times the requirement of the chicken. Further work is required to clarify this point.

Each egg laid by the three birds in each pen was analyzed for vitamin A and carotene and the values averaged for weekly

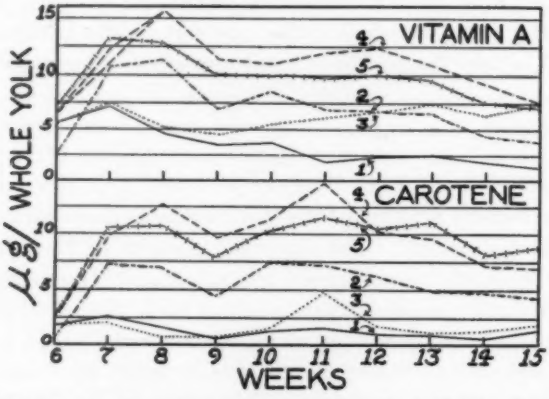

Fig. 3 Vitamin A and carotene content of egg yolk. 1. Basal ration. 2. Carotene supplement. 3. Vitamin A supplement. 4. Fresh alfalfa supplement. 5. Dehydrated alfalfa meal supplement.

periods, as is shown in figure 3. The values are given for the last 70 days of the experimental feeding period.

An analysis of variance showed highly significant differences between pens for the vitamin A and carotene content of egg yolk. The minimum difference required for significance (M. S. D.) between pens, for the vitamin A content of egg yolk, was 1.118 at the 0.01 level and 0.849 at the 0.05 level. For the carotene content of egg yolk the M. S. D. was 1.727 at the 0.01 level and 1.312 at the 0.05 level.

As is shown in figures 3 and 4, the carotene content of the egg yolk had been reduced to a low level at the end of the 33-

day depletion feeding period. The vitamin A content remained at a relatively higher level. On the 10th day of the experimental feeding period the first visible carotenoid pigments appeared in the petroleum ether extract of the egg yolk from the birds receiving alfalfa supplements. This first color was removed in the phasic separation of carotene, indicating the pigments to be carotenoids other than carotene. The carotene content of the egg yolk from the negative control pen, 1, gradually decreased during the 72-day experimental feeding period. The carotene content of the egg yolks from the birds receiving vitamin A was low, as would be expected.

Analysis of variance shows that, for the vitamin A content of egg yolk at the 0.01 level, pens 1, 2, and 3 each differ significantly from all the other pens. Pen 4 differs significantly from pen 5 at the 0.05 level. It will be noticed, in figure 3, that the eggs from pen 3 show a gradual increase in vitamin A content, while the general trend in the eggs from the other 4 pens is a decrease in vitamin A content. The vitamin A content of the eggs from pen 3 did not differ significantly from that of eggs from pens 4 and 5 at the termination of the experiment. The carotene content of the egg yolks, at the 0.01 level, from pens 1 and 3 each differs significantly from that of yolks from pens 2, 4, and 5, and pen 2 differs significantly from all the other pens.

The above differences in the vitamin A and carotene content of egg yolk further indicate that β-carotene in cottonseed oil is utilized less efficiently than are the vitamin A-active substances found in alfalfa. Further study is necessary to determine more accurately the extent of utilization of carotene as found in fresh alfalfa and dehydrated alfalfa meal. It is of interest to note that vitamin A from fish liver oil caused a definite increase in the liver reserves of vitamin A as contrasted to that from fresh alfalfa and dehydrated alfalfa meal, yet the alfalfa supported the higher egg yolk content of vitamin A. Furthermore, if all the carotene in the egg yolks from the birds receiving alfalfa is converted to vitamin A, the vitamin A value of the eggs would be approximately twice that of the eggs of

birds receiving carotene in oil, and three times that of the eggs from birds receiving vitamin A. It thus appears that alfalfa is more effectively utilized by chickens as a source of vitamin A-active substances in eggs than is vitamin A from fish liver oil, whereas the latter permits greatest liver storage.

Figure 4 shows the production record for the 105 days of the experiment.

There is normally a steady rise in egg production during the period in which the experiment was conducted. It will be noticed that at about 9 weeks there was a definite break in egg

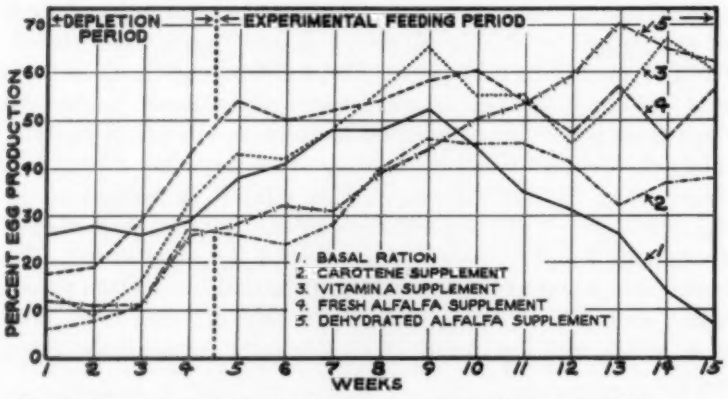

Fig. 4 Per cent egg production during the experimental period of 105 days.

production in the negative control pen, 1, and some indication of a break in pen 2. During the last week of the experiment egg production was 7% in pen 1, 38% in pen 2, 60% in pen 3, 56% in pen 4, and 62% in pen 5. The difference in egg production between pen 1 and pens 3, 4, and 5 appears to be significant. The difference between pen 2 and pens 3, 4, and 5 is indicative of significance. The sudden break in production at about 9 weeks supports the conclusions of McClymont and Hart ('48), who found that inadequate intake of vitamin A in white Leghorn pullets resulted in cessation of egg production at about 120 days, with death resulting at about 165 days. The

rate of egg production was affected only slightly before total cessation of production.

The syndrome of avitaminosis A was first observed at about the time of the break in egg production (9 weeks). The birds were droopy, refused food and water, and were in a badly emaciated condition. Multiple gray-colored retention cysts, each 1 to 2 mm in diameter, were found in the mucosa of the oesophagus and hard palate of most birds in pen 1. Enlarged ureters and kidneys filled with urates, a condition commonly associated with avitaminosis A in chickens, were not found as consistently as were the cysts in the oesophagus. The above conditions, together with sudden mortality, accounted for the termination of the experiment on May 18, 1948.

SUMMARY

Liver reserves of vitamin A in laying pullets partially depleted of their vitamin A and carotene reserves continued to decline when carotene equivalent to 2,000 I.U. of vitamin A activity per pound of ration was supplied in the form of carotene in cottonseed oil, fresh alfalfa, and dehydrated alfalfa meal. The carotene in oil was utilized less efficiently than that in the alfalfas. The carotene in fresh alfalfa was utilized somewhat more efficiently than that in dehydrated alfalfa meal. Vitamin A from fish liver oil, fed at the same level of vitamin A activity, enabled laying pullets to increase gradually their reserves of vitamin A in the liver.

The amounts of vitamin A and carotene in the eggs laid were greatest from pullets receiving the alfalfa supplements, while the next highest were from pullets receiving carotene in oil.

The total potential vitamin A activity in eggs from the pullets receiving the alfalfa supplements was about twice that of the eggs from birds receiving carotene in oil and three times that of birds receiving vitamin A from fish liver oil. Thus, vitamin A was superior in building liver reserves of the vitamin, while carotene from alfalfa, fresh or dehydrated, was

superior in transfer of vitamin A and carotene to the egg. Alfalfa appears to carry a factor which enhances the utilization of carotene.

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THE RELATIONSHIP OF DIETARY FACTORS TO THE TOXICITY OF ALLOXAN[1]

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It has been reported by Houssay and Martinez ('47) that dietary factors may influence the response of rats to alloxan. Mortality within 7 days following the injection was used as the criterion of the sensitivity of the animals. No data on blood sugar were presented but it was stated that in all cases marked hyperglycemia preceded death. Using as a base line a diet high in carbohydrate and low in fat, containing 20% protein, low protein or high lard or ox-fat caused increased mortality. Methionine or thiouracil (but not choline) supplementing the high lard diet overcame the deleterious effect of the fat. Animals receiving diets high in butter or olive oil responded in the same fashion as those on the low fat diet, while high oleomargarine, corn oil, or cocoanut oil had some beneficial effect. There is a similarity between the factors involved here and those which influence the development of massive hepatic necrosis (György, '44; Himsworth, '47). Of the group of substances which protect the liver against necrosis, only tocopherol (Schwartz, '44a, b; György, '47) is not mentioned. The present work was undertaken primarily to determine whether tocopherol might also protect the pancreas against the necrotic action of alloxan.

[1] Supported by a grant from Swift and Company, Chicago.

A major finding of this study, the extensive intravasal hemolysis and consequent high mortality within one or two days following the administration of alloxan to tocopherol-deficient rats, has been reported (György and Rose, '48). Further investigations in connection with this problem have been briefly presented (Rose and György, '49) and will be discussed in more detail in a later communication. The present report is concerned with the effect of tocopherol, as well as of some of the other dietary factors investigated by Houssay and Martinez, on the development of diabetes and on survival following alloxan injection.

EXPERIMENTAL

The general plan of the experiment followed that of Houssay and Martinez. Young female rats, weighing 90 to 135 gm, were given the experimental rations for about one month. At the end of this time they were fasted overnight and injected with 160 mg per kilogram of alloxan. Food intake was measured throughout the experimental period.

In the first study the animals were obtained from a local dealer. In all succeeding experiments the rats were of the Sprague-Dawley strain.

The experimental rations were of the type which had previously been used in producing hepatic injury, consisting insofar as possible of highly purified ingredients (table 1). The vitamin B complex was not incorporated in the diets but the crystalline vitamins (thiamine chloride, 20 μg; riboflavin, 25 μg; pyridoxine, 20 μg; and calcium pantothenate, 100 μg) were given daily as a separate supplement. Houssay and Martinez had used wheat and corn flours as the source of carbohydrate and yeast to supply vitamin B.

The experiments may be divided into three series. In the first experiment the dietary protein was low, as in studies of liver injury; in the second the protein level was raised to 20%, while in the third the 20% protein diets were modified by the addition of 5% of yeast, the regular vitamin B supplement not

being discontinued. A single group of animals in the second experiment was given a diet containing 40% of casein; a similar group in the third experiment received high protein and yeast (table 1).

TABLE 1

Composition of diets

DIET TYPE	CASEIN [1]	SUCROSE	FAT	COD LIVER OIL	YEAST	SALT MIXTURE
	%	%	%	%	%	%
Low protein, high fat	8	48	38	2		4
Low protein, low fat	8	88	[2]	[3]		4
Normal protein, high fat	20	36	38	2		4
Normal protein, high fat with yeast	20	31	38	2	5	4
Normal protein, low fat	20	76	[2]	[3]		4
Normal protein, low fat with yeast	20	71	[2]	[3]	5	4
Normal protein, high fat, no cod liver oil	20	76	40	[3]		4
High protein, high fat	40	16	38	2		4
High protein, high fat with yeast	40	11	38	2	5	4

[1] Vitamin test casein, General Biochemicals, Inc.
[2] Supplemented with three drops of corn oil daily.
[3] Supplemented with three drops of Mead percomorph oil weekly.

Three fats were studied: lard, hydrogenated cottonseed oil [2] and cocoanut oil. The high fat diets contained, in addition to 38% of the experimental fat, 2% of cod liver oil. The low fat ration contained no fat in the diet mixture, the animals being given supplements of three drops daily of corn oil to supply essential fatty acids and three drops weekly of percomorph oil for vitamins A and D. One group of animals on the high lard ration was given percomorph oil instead of cod liver oil to determine whether any benefit resulting from the low fat diet might be due to this substitution rather than the low level of dietary fat.

[2] "Vream," Swift and Company.

Methionine [3] (50 mg per day) and choline (25 mg per day) were fed with the vitamin B supplement. Tocopherol,[4] in the form of mixed natural tocopherols, was given either as a separate supplement of 3 mg per day dissolved in one drop of cottonseed oil or incorporated in the diet at a level of 50 mg per 100 gm to give approximately the same daily dose.

Alloxan [5] (160 mg per kilogram) was injected intraperitoneally, a freshly prepared solution containing 10 or 16 mg per milliliter being used. It was not possible to handle all of the animals of an experiment simultaneously, so one or two rats from each group were injected on the same day.

Blood sugar determinations [6] were made 48 hours after alloxan administration on all surviving rats, with a few exceptions where the condition of the animals made it impossible to obtain a blood sample. The animals were not fasted. Blood non-protein nitrogen (NPN) was determined at the same time on all animals of experiment II and half of those of experiment III. Sugar was estimated by the method of Somogyi ('37, '45) as modified by Nelson ('44), and NPN by an adaptation of the method of Folin and Denis ('16). Blood for both of these determinations was obtained from the tail.

The studies were first carried out with 10 rats in each group. All three experiments were then repeated so that most of the groups finally included 20 animals. In the second and third experiments there was no experimental difference between the two sections, so the values on the 20 animals of each group could be averaged together. In those groups which received the low protein diets (experiment I) there was a difference in the source of the rats in the two parts of the experiment. The very marked difference in the results of the two series required their separate consideration.

[3] Kindly furnished by Wyeth, Inc., Philadelphia.

[4] Kindly furnished by Distillation Products, Inc., Rochester, N. Y.

[5] Eastman Kodak Company.

[6] The blood sugar determination were made in the Cox Institute through the courtesy of Dr. F. D. W. Lukens.

TABLE 2

Effect of dietary factors on alloxan toxicity

GROUP	NO. OF RATS	BODY WEIGHT		BLOOD SUGAR			BLOOD NPN [1]	MORTALITY		FOOD INTAKE	
		Start	Change	No. of det'ns.	Average	No. of det'ns. less than 200 mg %		After 2 days	After 7 days	Before alloxan	After alloxan [2]
		gm	%		*mg %*		*mg %*	%	%	*gm/day*	*gm/day*
Experiment I-a: Low protein											
High lard	10	120	— 21	9	400	0		20	100	4.8	2.2
High lard + methionine	10	120	— 18	10	365	1		0	60	5.1	2.0
High lard + tocopherol	10	120	— 17	9	460	0		20	100	5.2	2.0
High Vream	9	122	— 17	8	390	1		0	67	5.5	3.5
High cocoanut oil	10	121	— 20	10	355	3		0	70	5.5	2.8
Experiment I-b: Low protein											
High lard	10	119	— 18	0				100		5.1	
High lard + methionine	10	118	— 8	0				100		5.3	
High lard + tocopherol	10	119	— 22	5	560	0		50	90	4.8	1.4
High Vream	9	119	— 18	7	440	2		0	67	5.6	2.2
High cocoanut oil	9	122	— 18	2	440	0		90	100	5.2	1.1
Low fat	10	118	— 8	4	305	2		50	50	8.1	7.6
Low fat + tocopherol	10	121	— 9	5	355	2		30	60	8.7	4.7
Experiment II: "Normal" protein											
High lard	20	110	+ 6	10	485	0	205	55	95	5.0	0.9
High lard + methionine	20	108	+ 43	10	370	3	155	50	90	5.8	1.2
High lard + choline	20	108	+ 36	12	410	3	125	35	75	5.9	2.9
High lard + tocopherol	20	109	+ 15	15	490	0	90	25	85	5.3	2.4
High lard + tocopherol [3]	0	108	+ 8	6	475	0	80	33	100	4.3	1.7
High Vream	20	109	+ 25	18	510	2	110	10	65	5.7	2.8
High cocoanut oil	20	109	+ 2	9	280	3	175	60	80	4.9	2.1
High cocoa. oil + tocoph.[3]	10	106	— 4	7	330	2	95	20	80	3.7	1.5
High lard without cod liver oil	10	112	+ 12	2	430	0	170	80	100	5.5	2.1
Low fat	20	108	+ 23	14	660	2	170	25	55	8.0	3.2
Low fat + tocopherol	20	106	+ 26	18	520	4	105	0	30	7.7	6.3
High lard + high protein	20	108	+ 24	5	400	0	215	75	95	4.9	1.7
Experiment III: "Normal" protein + yeast											
High lard	20	107	+ 41	17	560	1	120 [4]	15	90	6.3	1.8
High lard + methionine	20	106	+ 57	15	550	1	145	35	80	6.5	2.2
High lard + choline	20	107	+ 48	14	450	2	110	35	85	6.8	1.8
High lard + tocopherol	20	109	+ 47	20	455	0	95	0	60	6.5	2.5
High lard + tocopherol [3]	20	108	+ 49	17	500	1	90	15	85	6.7	3.1
High lard + tocoph.[3] + methionine	20	108	+ 64	20	540	1	90	0	75	7.1	3.6
High Vream	20	108	+ 53	19	700	1	170	20	95	6.7	2.1
High cocoanut oil	20	108	+ 35	18	340	6	85	15	55	6.4	2.2
High cocoa. oil + tocopherol [3]	20	107	+ 36	19	385	3	115	5	55	6.4	3.0
Low fat	20	108	+ 47	18	515	2	140	5	20	9.7	5.4
Low fat + tocopherol	20	107	+ 52	18	610	2	130	5	35	10.3	5.1
High fat + high protein	20	108	+ 58	17	435	3	115	30	65	6.3	2.1

[1] Average NPN before treatment with alloxan = 50 mg %.
[2] Average intake for two to 4 days following alloxan injection in rats with diabetes.
[3] Tocopherol mixed with the dietary fat.
[4] In experiment III the NPN was measured on half of the animals of each group.

In our experiments the proportion of the rats dying within 7 days after injection with alloxan was not an entirely satisfactory criterion for estimation of the influence of the dietary factors studied. It made no distinction between the groups in which the majority of the animals died during the first day or two after the injection and those in which the deaths were spread over 5 or 6 days. Since this difference proved to be of significance, mortality within two days after the administration of alloxan as well as during the first week has been tabulated.

RESULTS

Tocopherol was found to be the most important factor involved in preventing early mortality. The effect of tocopherol was the same whether it was fed as a separate supplement or was mixed with the lard or cocoanut oil of the diet. The same favorable response was observed in animals which received diets containing an hydrogenated cottonseed oil of which tocopherol is a natural constituent. An explanation of the high early mortality of the tocopherol-deficient animals was found in the hemolysis which has been described previously (György and Rose, '48). Hemolysis was never observed in any animals treated with tocopherol. This all-or-none response could not be duplicated in the death rate, since the toxicity of alloxan was not due solely to hemolysis. In most of the individual groups the mortality of the animals treated with tocopherol was definitely lower than that of those deficient in tocopherol, and when larger units were considered the differences were unquestionable. So, in the series receiving the diets containing 40% of fat and 20% of casein, mortality during the first two days after injection in all of the tocopherol-deficient groups combined was 51%, in the tocopherol-treated groups, 16%. In only one experiment (Ia) was the mortality in the groups which had not received tocopherol low during this initial two-day period. These animals were the only ones of the whole series which were not obtained from Sprague-Dawley, and they may have received enough tocopherol during the pre-experimental

period to protect them against depletion during the month on the tocopherol-deficient diet.

The protective effect of tocopherol seemed to be limited to the period immediately following administration of alloxan. At the end of a week the tocopherol-treated groups had almost overtaken the deficient ones in the number of deaths. In the series referred to above, namely the groups receiving diets high in fat and with normal content of casein, mortality of the tocopherol-deficient animals within 7 days was 87%; of the tocopherol-treated ones, 79%.

Yeast showed an effect on survival similar to but less pronounced than that of tocopherol. When 5% of yeast was added to the high fat rations, mortality was reduced from 51% to 25% during the first two days, but after 7 days 79% of the animals were dead. Tocopherol with yeast lowered the death rate during the early period to 8%, but even with this combination 74% of the animals died within one week.

Variation of the protein content of the diet had little effect on survival. Sprague-Dawley rats receiving only 8% of casein were somewhat more susceptible to the effects of a deficiency of tocopherol immediately after injection with alloxan than those on the diet containing 20% of casein. When the 7-day period was considered, however, there was as high mortality with 20% and even with 40% of casein as with 8% in the groups which had received diets with a high content of fat. With low-fat diets there was a slight, but statistically insignificant, difference in favor of a normal over a low level of protein.

Methionine and choline, substances which have a specific protective effect on the liver, were quite without influence on the toxicity of alloxan. Mortality during the two-day and the 7-day period was as high as with the unsupplemented diet. Methionine in conjunction with tocopherol gave no advantage over tocopherol alone.

Few rats receiving high fat diets survived more than 7 days. Mortality was low during the first two days when the hydro-

genated cottonseed oil was used, because it contained tocopherol. There was a slight advantage shown by cocoanut oil over lard or cottonseed oil at the end of 7 days in those groups which had received yeast, but not in those without yeast.

When fat was eliminated from the diet the toxicity of alloxan was much reduced. Only with this type of diet did an appreciable proportion of the rats survive more than 7 days after the injection. The average survival for this period of all the animals on the low fat diets was 61%, while the average of all the high fat groups was 19%.

Most of the animals injected with alloxan became diabetic regardless of the nature of the diet which they had received. The average level of blood sugar 48 hours after alloxan was given was high in all groups, ranging from 280 to 700 mg per 100 ml. In view of the wide range of the individual values, no statistical significance can be assigned to the group averages. Arbitrarily designating a blood sugar level exceeding 200 mg per 100 ml as an indication of the presence of diabetes, a slightly lower incidence of the disease was observed in animals receiving diets low in fat or containing cocoanut oil. With high lard or high cottonseed oil content, 94% of the animals surviving more than 48 hours had diabetes; with low fat diets, 86%; and with high cocoanut oil diets, 81%. These differences, though small, are significant ($P < 0.001$).

The general picture in almost all animals which survived more than 7 days was a gradual improvement in the diabetes. Blood sugar values were lower after several weeks, and fasting sugar values almost normal. Most of the animls showed persistent moderate to severe glycosuria. Polyuria and glycosuria were especially prominent in the rats receiving the low fat (high carbohydrate) diet.

Blood non-protein nitrogen, measured 48 hours after injection of the animals, was elevated in almost all cases (György and Rose, '49), the highest values being found in the tocopherol-deficient groups. The kidneys of most of the animals were examined histologically and the same type of damage

found in tocopherol-treated and tocopherol-deficient groups.[7] The higher NPN of the tocopherol-deficient animals was probably a consequence of the blocking of the kidneys with hemoglobin from the damaged red blood cells.

DISCUSSION

Under the conditions of our experiments the dietary factors studied seemed to be of little significance with respect to the diabetogenic effect of alloxan. The premise that there are similar protective agents effective against necrosis of the pancreas and liver was not sustained. Methionine, choline and tocopherol were equally ineffective in preventing elevation of the blood sugar level following alloxan administration. Elimination of fat from the diet resulted in only a small reduction in the incidence of diabetes. There was a slightly greater effect when cocoanut oil was introduced into the diet, but even in this case 81% of the animals were diabetic.

The significance of the blood sugar values as an indication of diabetes may be lessened to some extent by two factors, kidney damage and food intake. That the former was without appreciable effect may be seen by the similarity of the average figures for blood sugar in the tocopherol-deficient groups, where nitrogen retention was generally large, and in the tocopherol-treated groups, which showed only moderate retention. Food intake after the injection was low in all groups but somewhat higher in those given the low fat than in those receiving the high fat rations. The elevation of the blood sugar values may have been exaggerated somewhat in the low fat groups, but the persistent excretion of large volumes of urine with a high content of sugar gave additional evidence of severe damage to the pancreas.

[7] Microscopic examination of a large number of kidneys and livers obtained from our animals revealed severe acute tubular changes in the kidneys and often intensive parenchymatous (hydropic) degeneration in the liver. There was no indication of fatty degeneration, and glycogen stains showed, if anything, less than a normal content of glycogen. We are indebted to Dr. Harry Goldblatt, Institute for Medical Research, Los Angeles, for these findings.

Mortality within 7 days was the criterion used by Houssay and Martinez ('47) in determining the severity of the effect of alloxan. We were unable to duplicate their finding that methionine would reduce mortality after alloxan administration in rats receiving a diet high in lard. Even with 50 mg per day of methionine most of the animals died within 7 days. We did observe much better survival with low fat than with high fat diets. Houssay and Martinez found replacement of lard with cocoanut oil even more effective than its replacement with carbohydrate, mortality being reduced from 100% to 0%. In our experiments, in only two of the 6 pairs of groups differing only as to whether they were fed lard or cocoanut oil as the dietary fat was there a significant reduction in the number of deaths. Considering the 6 pairs collectively the difference is of definite mathematical significance, though of less importance practically since mortality during the first week was still 69% as compared with 93% for the animals receiving lard and 35% for those on the low fat diet.

Although recent interest in alloxan has been concentrated on its ability to produce diabetes, many other toxic effects have been reported. Its action as a capillary poison and spasmodic was studied by Labes and Freisburger ('30) and the severe kidney damage following its injection has been described many times (compare Lukens, '48). In the present paper we have discussed the hemolyzing effect of alloxan in tocopherol-deficient rats. In our experiments diabetes did not seem to be primarily responsible for the death of the animals. Mortality and diabetes were decreased to about the same extent when cocoanut oil was fed, but the effect was too small to allow any conclusions to be drawn from it. Only rats receiving diets low in fat had an appreciably prolonged survival, and in most of these diabetes, as measured by blood and urine sugars, was severe. Nor, in these animals, was nitrogen retention less than in other groups. The response of some other tissues to the action of alloxan must be modified by the feeding of fat or carbohydrate.

An explanation has been offered for the protective effect of tocopherol (György and Rose, '49). In its absence, either blocking of the kidneys with hemoglobin or anemia may be in part responsible for the death of the animal, or may weaken its resistance to the other toxic effects of alloxan. It has been found that, as far as hemolysis is concerned, tocopherol acts specifically in the red blood cell, presumably through its anti-oxidant activity (Rose and György, '49). The mode of action of yeast is not so definite. Animals receiving yeast were better nourished than those without it, but the greater effectiveness of yeast with respect to survival in the absence of tocopherol than when fed as a supplement to it, and the failure of protection after 7 days, suggest that its action may be similar to that of tocopherol. Hemolysis was not prevented completely by yeast as it was by tocopherol, but it was reduced in incidence and severity. Yeast contains anti-oxidants (György and Tomarelli, '43) and these may be effective in reducing the toxicity of alloxan.

The two experiments with the low protein diet, in which rats obtained from different sources were used, show how much care must be used in the interpretation and general application of the results of experiments of this type, particularly in studies of such short duration. In the first study there was almost no mortality in the tocopherol-deficient groups during the first two days after alloxan administration; in the second, very high mortality figures were recorded. The probable explanation of this discrepancy is a difference in tocopherol intake during the pre-experimental period. The data indicate that with the first rats used the reserve of tocopherol was sufficient that the animals were not depleted by one month on the tocopherol-deficient diet, and consequently neither the very severe symptoms of the latter group nor any benefit of tocopherol were observed.

SUMMARY

The influence of dietary factors on the sensitivity of rats to alloxan has been investigated. The diet was varied with re-

spect to its content of protein and fat, and supplements of methionine, choline, tocopherol, and yeast were given.

The toxicity of alloxan, as measured by the number of rats which survived for 7 days after its injection, was decreased appreciably only when diets very low in fat were fed. There was a slight advantage shown by cocoanut oil over lard or hydrogenated cottonseed oil in the high fat diets. The hemolysis which occurred when alloxan was administered to tocopherol-deficient rats caused a high death rate during the first two days after the injection. Tocopherol and, in an apparently somewhat similar fashion, yeast, reduced the early mortality but had no effect after this initial period.

Diabetes developed in most of the animals regardless of the nature of the diet. Considering those animals as diabetic whose blood sugar, 48 hours after injection with alloxan, was above 200 mg per 100 ml, the incidence of diabetes was slightly, but significantly, lower with diets low in fat or containing cocoanut oil than with those containing lard or hydrogenated cottonseed oil. There was no evidence of any benefit from any other of the variations in diet studied. Even tocopherol, in spite of its very specific effect in preventing hemolysis following the injection of alloxan, gave no protection against the diabetogenic effect of alloxan.

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THE VITAMIN A REQUIREMENT OF GROWING TURKEYS [1]

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THREE FIGURES

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Investigations of the vitamin A requirement of growing turkeys have produced diverse results. Scott and Hughes ('32), using a diet containing white corn, ground wheat, wheat bran and meat and bone scraps with yellow corn as the source of vitamin A, reported that 50 or 60% of the ration should consist of yellow corn to provide the minimum requirement of vitamin A for growing turkeys. This amounts to approximately 350 to 420 I.U. of vitamin A per 100 gm of feed. Hinshaw and Lloyd ('34) reported 1,736 I.U. of vitamin A per 100 gm of feed as the minimum requirement. Their diet contained white corn, barley, wheat, fish scrap, dried milk, bone meal, ground limestone and salt, with alfalfa leaf meal as the source of vitamin A. From a nutritional standpoint this diet is an improvement over the one used by Scott, yet, fortified with alfalfa leaf meal at the level reported as optimum it produced birds averaging only 1,200 gm approximately at 12 weeks of age. Wilgus ('40), using a more complete diet

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including soybean oil meal, casein, dried brewers' yeast, manganese sulfate and irradiated sterol, with crystalline carotene as the source of vitamin A, reported 600 I.U. of vitamin A per 100 gm of feed as meeting the requirements of growing turkeys. Weights at 8 weeks of age at this level averaged 1,033 gm. Scott, Matterson and Jungherr ('45–'46) obtained average weights of 1,376 gm at 8 weeks of age on a vitamin A deficient diet fortified with 1,000 I.U. of vitamin A per 100 gm of feed, and concluded that this level of vitamin A was optimum. The National Research Council ('44) recommended an allowance of 551 I.U. per 100 gm of feed.

A serious fault with previously conducted investigations to determine the vitamin A requirement of growing turkeys has been the poor growth obtained on the diets used. A casual examination of most of these diets clearly reveals that many were deficient in the B complex vitamins. It appears that factors other than vitamin A limited the growth in most, if not all, of these experiments.

EXPERIMENTAL

The diet used in the experiments reported herein (table 1) was an improved modification of the 1946 Connecticut vitamin A "free" turkey diet. The experimental diet was supplemented with all the known crystalline B vitamins at or above the level recommended by the National Research Council ('44, '46). Where requirements have not been determined, the vitamins were added at a level indicated as sufficient by previous experiments conducted at the Storrs Agricultural Experiment Station (unpublished data). This diet, properly fortified with vitamin A, produced poults averaging over 2,000 gm at 8 weeks of age.

A basal diet containing all the ingredients except white corn was mixed in amounts deemed sufficient to last for the duration of each experiment, although in one case it was necessary to mix an additional amount.

A premix of white table corn meal and cod liver oil [5] assayed at 2,225 I.U. of vitamin A per gram was substituted for white table corn meal in the basal ration to provide experimental diets containing 0, 150, 300, 600, 1,200, 2,400, 4,800 and 7,200 I.U. of vitamin A per 100 gm of feed for lots 1 through 8, respectively. The premix and individual lot diets were mixed at intervals of 10 days and stored at — 10°C.

In the first two experiments 161 unsexed day-old Broad Breasted Bronze poults from the college flock were used. These poults were divided into weight groups covering a

TABLE 1

Composition of the vitamin A "free" ration

INGREDIENT	AMOUNT	INGREDIENT	AMOUNT
	lb.		
White table corn meal	53.4	*Vitamin supplement/100 lb.*	
Acid precipitated casein	20.0	Choline chloride	104.00 gm
Soybean oil meal	15.0	Nicotinic acid	2.27 gm
Butyl fermentation solubles [1]	4.0	2 Methyl-naphthoquinone	45.00 mg
Wheat germ oil	2.0	Riboflavin	227.00 mg
Salt	0.5	Ca pantothenate	635.60 mg
Ground limestone	1.0	Thiamine	90.80 mg
Bone meal	4.0	Inositol	227.00 mg
Activated animal sterol		Para-aminobenzoic acid	605.00 mg
200 D/gm	0.1	Pyridoxine	227.00 mg
$MnSO_4$	0.025		

[1] Curbay B G "80."

range of 5 gm. Equal numbers of poults from each weight group were selected at random to make up the necessary lots. The poults were wing banded and weighed and individual weights were taken weekly for the duration of the experiment.

The 188 poults used in experiment 3 were male day-old Broad Brested Bronze poults obtained from a commercial hatchery. They were divided into lots by the method described above and the experiment was replicated three times. Birds in replicate 3 were used for laboratory tests.

All poults were kept in a standard Petersime electric battery brooder and at 4 weeks of age were moved to new locations

[5] Kindly supplied by Dr. John Correll of the Upjohn Company, Kalamazoo, Michigan.

in standard developing batteries. Replicated experiments were arbitrarily assigned a battery for each replicate and the location of each lot was chosen at random.

Two birds from each lot, experiment 3, replicate 3, were sacrificed biweekly, beginning at the second week, for vitamin A and carotene determinations on blood and liver.

The method of Kimble ('39) was essentially followed in extracting vitamin A and carotene from the blood samples. The saponification of liver samples and the extraction of vitamin A followed the method of Davies ('33). The activated 1–3 glycerol dichlorohydrin method of Sobol and Werbin ('45) was used for the determination of vitamin A.

Blood samples were secured by bleeding from the heart and determinations were made the same day the samples were drawn. The liver samples were refrigerated under potassium hydroxide until the analysis could be made. Beak and brain sections were taken for histological study.[6]

Experiment 1 was started May 3 and contained 7 lots, replicated twice. This experiment was closed when the birds were 6 weeks of age. Experiment 2, a duplicate of experiment 1, was started May 17. The poults in experiment 3, started June 24, were divided into three replicates. A negative control (lot 1) was established in replicate 3 only and an additional level of vitamin A (lot 8) included. Experiments 2 and 3 were carried until the poults were 8 weeks of age.

At the close of each experiment the sex of each bird was established. An incision was made in the dorsal region between the 6th and 7th rib, exposing the abdominal cavity, and the sex was determined on the basis of the presence or absence of a testicle.

All birds were allowed ad libitum feeding and feed consumption records were kept.

The growth data analysis was based on a modification of the Wishart ('39) method. The basic principal in this method involves analysis based on constants used in fitting the weight

[6] Histological interpretations were made by Dr. E. Jungherr of the Animal Disease Laboratory, Storrs Agricultural Experiment Station.

observations of each animal to a curve. This method allows the use of the original data, and differences in the response over the entire experimental period can be measured.[7]

The logarithms of the weights were used in this analysis since, according to Wishart, growth differences can be elucidated by examination of one or two constants of the curves fitted to the logarithm of the weights.

Two terms, a linear and a quadratic, were used to fit the logarithm of the weights of the male poults to a curve. Graphical examination of these coefficients revealed that the linear coefficient, representing the slope of the curve, was related to dosage of vitamin A; and that the quadratic coefficient, descriptive of the departure from linearity, was not related to treatment. Subsequent analyses were then made on the linear coefficient.

The mean, adjusted coefficients were fitted to a curve by the method of covariance with two concomitant measures, and plotted against log-dose. This method allows the number of individuals in each lot to influence the derived or calculated point on the curve it will occupy, and enables the calculation of the point of greatest response to treatment if the range of treatment is sufficient to reach a point of plateau of diminished response.

RESULTS

Growth

Figure 1 shows the adjusted mean linear coefficients plotted against the log-dose as the abscissa. The response to increased levels of vitamin A up to 6 weeks of age (expt. 1) can be compared with the response up to 8 weeks of age in experiments 2 and 3. The calculated point of greatest response is at levels of 1,760, 1,512, and 1,776 I.U. of vitamin A per 100 gm of feed for experiments 1, 2 and 3, respectively.

The negative controls in all experiments died by the end of the 4th week. All were found to have gross evidence of

[7] Details of the statistical treatment of lots will be published in expanded form in a forthcoming Experiment Station bulletin.

A-avitaminosis. While ataxia was a fairly constant observation, many birds died suddenly without evidencing any gross pathological symptoms.

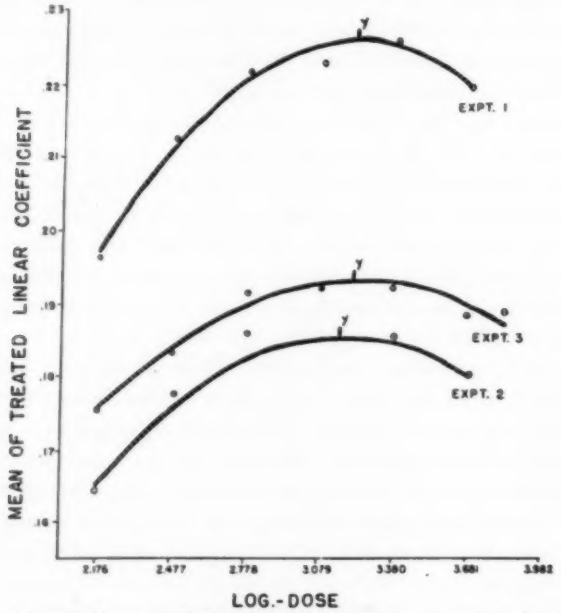

Fig. 1 Average linear coefficients for experiments 1, 2 and 3 plotted against concentration of vitamin A in the diet together with calculated point of greatest response.

Blood levels of vitamin A

A rapid rise in the concentration of vitamin A in the blood with increases of vitamin A in the diet to the 1,200 I.U. per 100 gm of feed level is shown by the 6-week data, which is plotted graphically in figure 2. Beyond this point blood vitamin A concentration more or less reaches a plateau.

Liver storage of vitamin A

In all lots receiving less than 1,200 I. U. of vitamin A per 100 gm of feed, liver stores of vitamin A decreased between

the second and 4th week. At this point lots 2 and 3 reach a plateau and lot 4 shows a slight increase in storage (fig. 3). Liver storage at two weeks of age was significantly higher in the birds of lots 5, 6, 7 and 8. Lot 5 (1,200 I. U.) maintained its liver stores over the experimental period, and lots 6, 7 and 8 substantially increased their liver stores with age.

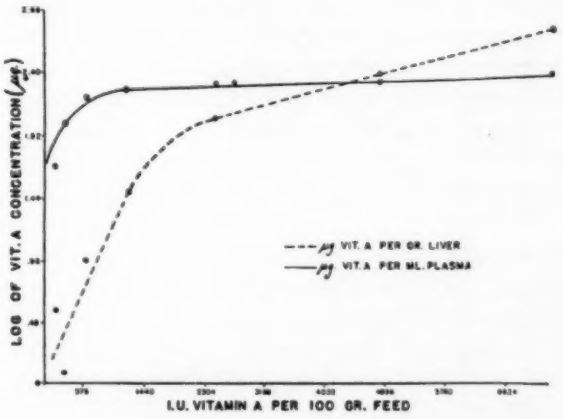

Fig. 2 Concentration of vitamin A in blood plasma and liver for poults at 6 weeks of age on increasing levels of vitamin A in the feed.

Histopathology

No nasal lesions were observed in any bird receiving 150 I.U. of vitamin A per 100 gm of feed or amounts above this, nor were lesions found in any birds at two weeks of age. Of the 9 poults started in the negative control group, only one survived to 4 weeks of age and in this bird only were the characteristic lesions of A-avitaminosis found.

Lesion in the brain stem and optic chiasma which, according to Adamstone ('47), are specific for vitamin A deficiency in chickens, occurred in turkeys 6 and 8 weeks of age on all levels of vitamin A. No lesions were found in any bird under 6 weeks of age receiving a vitamin A supplement.

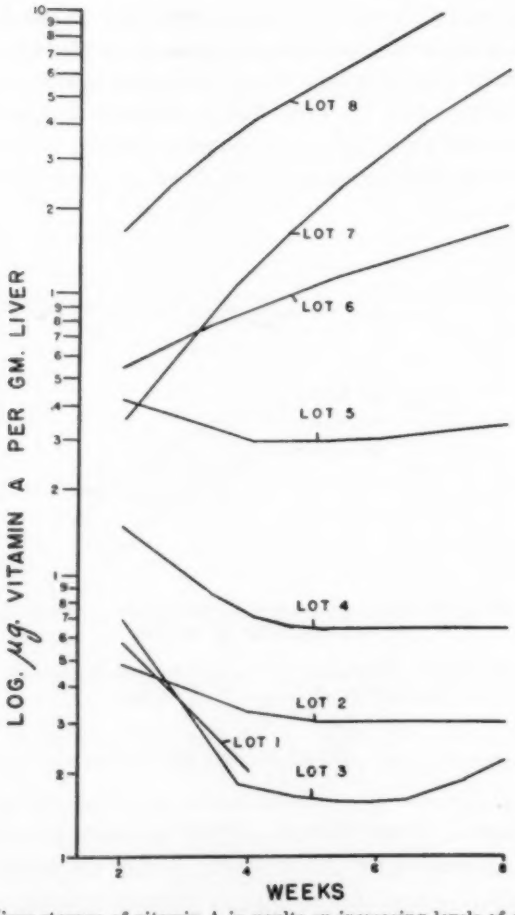

Fig. 3 Liver storage of vitamin A in poults on increasing levels of vitamin A in the diet plotted against time.

DISCUSSION AND CONCLUSIONS

There is evidence to indicate that males have a higher vitamin A requirement than females (Scalongne, '40; Brenner, Banks and Roberts, '42; Popper and Brenner, '42). On this premise it was reasoned that data secured from the male poults

would represent the most valid basis for determining the vitamin A requirement of the turkey poult. Therefore only the male birds in the three experiments reported herein were used as a source of growth data.

The use of growth as a criterion for the measurement of the vitamin requirement of an animal does not preclude nor invalidate the use of such criteria as longevity, lactation, egg production, et cetera. It does, however, assume that maximum growth is optimum growth; thus the minimum requirement for any nutrient for growth may be defined as the lowest level of that nutrient which allows the maximum growth inherently possible. This definition describes equally well the maximum requirement because, according to it, additional increments of the nutrient fail to induce additional response in growth.

The usual statistical treatment of growth data of this type is to make an analysis of variance in weight at a given age and of final weight or net gain in weight of the experimental lots, and to make T tests between lots. The level at which T values cease to be significant is concluded to represent the minimum requirement. This is not a valid statistical method because it involves segmentation of the data, and the differences, which with finite numbers are insignificant, become significant when infinite numbers of experimental animals are used. In the present paper, maximum and minimum requirements for growth are considered synonymous and the level of vitamin A reported as inducing the greatest growth response may be considered the minimum or maximum amount required by growing turkeys.

With this in mind it is possible to calculate the point of greatest response to vitamin A, which, as is shown in figure 1, is at the level of 1,760, 1,512, and 1,776 I.U. of vitamin A per 100 gm of feed for the three experiments, respectively. The average of these is 1,683 I.U. of vitamin A per 100 gm of feed.

From these data it is concluded that 1,700 I.U. of vitamin A per 100 gm of feed represents the optimum level of this

vitamin for growing turkeys. This corresponds well with the results of Hinshaw and Lloyd ('34), who reported 1,736 I.U. of vitamin A per 100 gm of feed as meeting the minimum requirement of growing turkeys, and is far above the recommendations of other investigators.

No conclusive results were obtained from the histological examination of the beaks.

As is shown in figure 2, the concentration of vitamin A in the blood plasma of birds 6 weeks of age increased as the level of vitamin A in the diet was increased. This increase was quite rapid between lots 2 and 4, reaching more or less a plateau in lot 5, which received 1,200 I.U. of vitamin A per 100 gm of feed. A comparison of liver storage among birds in the 8 experimental lots is shown in figure 2. Liver storage of vitamin A increased rapidly between lots 2 and 5, and at a diminishing rate thereafter.

Figure 3 shows the average concentration of vitamin A per gram of liver, by lots, for birds two, 4, 6 and 8 weeks of age. Lots receiving less than 1,200 I.U. of the vitamin per 100 gm of feed showed a depletion of liver stores between the second and 4th week, with liver concentration of vitamin A remaining at a fairly constant level thereafter. Lot 5, except for a slight drop at 4 weeks, maintained its liver vitamin A at about the two-week level. Increased storage of vitamin A with increased age was found only in lots 6, 7 and 8, the amount of storage increasing as dosage was increased.

These data are very much in accord with the results of the analysis of the growth data. Plasma vitamin A concentration reaches a plateau when 1,200 I.U. of the vitamin per 100 gm of diet are fed, the amount given lot 5, and liver stores of vitamin A are maintained in a steady state at this level. Lots receiving less than 1,200 I.U. of vitamin A per 100 gm of feed responded to increases of vitamin A in the diet with large increases in the blood plasma level of the vitamin. These lots were also unable to maintain their stores of vitamin A in the liver. Additional increases of vitamin A in the diet, above 1,200 I.U., resulted in insignificant increases in the plasma level of vita-

min A but enabled the birds to store the vitamin in the liver at a rate proportional to the increased dose.

This suggests a minimum physiological requirement of turkey poults, as applied to liver storage and plasma levels, of 1,200 I.U. of vitamin A per 100 gm of feed, with an indicated optimum between 1,200 and 2,400 I.U., which optimum correlates well with the 1,700 I.U. of the vitamin which the growth data reveal as the level inducing the greatest response.

SUMMARY

1. Three experiments were conducted in which 228 male Broad Breasted Bronze turkeys were used to determine the vitamin A requirement of growing turkeys, the experiments being so designed as to allow the application of three criteria: growth, histopathology, and vitamin A concentration in the blood and liver.

2. A basal vitamin A deficient diet was used which, when adequately supplemented with vitamin A, was capable of producing birds averaging 2,000 gm in weight at 8 weeks of age. This diet was supplemented with levels of 0, 150, 300, 600, 1,200, 2,400, 4,800 and 7,200 I.U. of vitamin A per 100 gm of feed.

3. From the analysis of growth curves, the point of greatest response was calculated to be 1,683 I.U. of vitamin A per 100 gm of feed.

4. Histological examination of the brain revealed no conclusive results.

5. The plasma level of vitamin A in lots receiving less than 1,200 I.U. of the vitamin per 100 gm of feed was greatly influenced by the dosage of vitamin A in the diet. Lots receiving more than 1,200 I.U. of vitamin A per 100 gm of feed showed little effect of the dosage on plasma levels.

6. Lots receiving less than 1,200 I.U. of vitamin A per 100 gm of feed were unable to maintain their liver stores of the vitamin with increased age, while lots receiving more than 1,200 I.U. per 100 gm of feed increased their liver stores as age advanced.

7. It is concluded on the basis of the growth data and the plasma levels and liver storage of vitamin A that the vitamin A requirement of growing turkeys is 1,700 I.U. of the vitamin per 100 gm of feed.

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NITROGEN BALANCE IN MEN CONSUMING RAW OR HEATED EGG WHITE AS A SUPPLEMENTAL SOURCE OF DIETARY PROTEIN [1]

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Reports by Schwimmer and associates ('47, '48) indicate that for men consuming diets low in calories (approximately 1,800 Cal. daily) and drinking small quantities of water (800 ml daily), dried egg white provides an excellent source of available nitrogen. Because of this, it was decided to incorporate dried egg white as a major source of protein into certain experimental food items for trial by the Armed Forces. However, it was questionable whether the moderate degree of heat that could be applied in preparation of these items would be sufficient either in degree or duration to destroy the anti-trypsin which is present in commercial egg white powder. This possibility seemed to be a real one in view of Harte's observations ('45). He found by *in vitro* investigations that commercially dried egg white contained as much anti-tryptic principle as undried egg white, and that the digestibility of these products was inferior to that of coagulated egg white as determined by *in vitro* measurment.

In the present communication we report observations made to determine the importance of egg white anti-trypsin in human

[1] The opinions expressed in this paper are those of the authors, and do not necessarily represent the official views of any governmental agency.

nutrition. Nitrogen balance was measured in men who consumed the following proteins as supplemental sources of dietary nitrogen: (a) lactalbumin; (b) unheated commercial dried egg white; (c) heated commercial dried egg white; and (d) heated commercial dried egg white plus its natively equivalent amount of chemically isolated anti-trypsin (ovomucoid). The last named substance was studied in an attempt to determine specifically the relationship between the naturally occurring anti-trypsin and the chemically isolated form.

METHODS

General

Two healthy, young, volunteer, male subjects were studied for approximately 6 weeks in a metabolic ward in a research hospital. The subjects were allowed freedom of activity in the ward. One subject worked in a hospital laboratory during the day but was not physically very active. The other was allowed to leave the hospital three times a week to visit friends but exercise was kept at a minimum. Temperature, pulse, respiration, body weight, and basal blood pressure were recorded each day, as was the general state of health. Special care was taken to note any symptoms or reactions that might be related to consumption of the diets. All urine and stools (with carmine markers) were collected and stored under refrigeration. The 24-hour urine output was recorded, three-day composite specimens were prepared with toluene (2 ml per 100 ml) as the preservative, and aliquots were taken for analysis of total nitrogen. The stools were mixed in a Waring Blendor with distilled water and aliquots taken for total nitrogen determination.

Diet and fluid intake

Each subject subsisted on a fixed basal diet and a constant fluid intake (2,500 ml/day). The basal diet consisted of 4 well varied menus of natural foods; each menu was rotated in the same sequence during each of the 5 experimental periods described below. An example of one of the menus follows:

Breakfast: orange juice; applesauce; puffed rice and cream; bread, butter and cinnamon sugar; beverage. *Dinner:* apple juice; Spanish rice; broccoli, frozen; fresh vegetable salad with dressing; olives; crackers, butter and jelly; orange ice; and beverage. *Supper:* pinapple juice; vegetable salad; corn, canned; beets, canned; green beans, canned; tomato, fresh; lettuce and pickle, with dressing; crackers, butter and jelly; Jello and banana; and beverage. All food items were carefully weighed and prepared by the dietitian. There was no plate waste.

The basal diet was supplemented with 250 μg of biotin and one multiple vitamin capsule daily. Each capsule provided: thiamine, 2.0 mg; riboflavin, 2.0 mg; niacinamide, 10.0 mg; pyridoxine, 0.2 mg; calcium pantothenate, 3.0 mg; choline, 20.0 mg; inositol, 10.0 mg; folic acid, 10 μg. The basal diet was calculated to contain the following as an average: Calories, 2,447; protein, 26.9 gm; fat 88.6 gm; carbohydrate, 385.6 gm; iron, 11.5 mg; vitamin A, 13,076 I.U.; thiamine, 3.0 mg; riboflavin, 3.7 mg; niacin, 19.0 mg; vitamin C, 265 mg. Duplicate meals from each of the 4 menus were analyzed for nitrogen content; this was done in each period described below. The test proteins were added to drinks and cooked dishes in such proportions as to be almost undetectable. The amounts used were as follows:

Period I (9 days): Unheated lactalbumin, 30 gm (66.25% protein).

Period II (9 days): Unheated commercial egg white power, 25.8 gm (77.5% protein).

Period III (9 days): Heated commercial egg white powder, 25.8 gm (77.5% protein).

Period IV (9 days): Heated commercial egg white powder, 25.8 gm (77.5% protein) plus ovomucoid 4 gm.

Period V (6 days): Unheated lactalbumin 30 gm (66.25% protein).

The total protein intake was therefore approximately 50 gm per day. It will be noted that the periods when anti-trypsin was present were alternated with periods when it was absent.

This arrangement tended to eliminate any "metabolic lag" that might arise due to the former period.

Some results of studies on the *in vitro* digestibility of the test proteins are shown in table 1.

It is apparent that the *in vitro* anti-tryptic activity of the dried egg white was high, and that wet autoclaving was necessary to cause improvement in digestibility to that of lactalbumin. This method was therefore used in preparing the heated egg white for periods III and IV. The ovomucoid used in period IV was prepared according to the method described by Lineweaver and Murray ('47). That this substance also had high *in vitro* anti-tryptic activity is shown by data presented in table 2.

TABLE 1

Studies on the in vitro digestion [1] *of egg white and lactalbumin by trypsin*

DAY OF DIGESTION	UNHEATED COMMERCIAL EGG WHITE POWDER % Digestion	COMMERCIAL EGG WHITE POWDER, AUTOCLAVED DRY % Digestion	COMMERCIAL EGG WHITE POWDER, AUTOCLAVED WET % Digestion	POWDERED LACTALBUMIN, UNHEATED % Digestion
1	2.8	3.7	30.0	42.8
4	5.5	6.2	26.6	29.0
5	5.0	7.8	24.1	25.6
6	6.1	7.1	23.8	25.1
7	6.2	6.5	22.7	22.9

[1] Per cent digestion $= \frac{\text{increase in titratable amino N} \times 100}{\sqrt{10.15 \text{ days} \times \text{gm} \times 0.01 \times \% \text{ protein}}}$. See Freed et al. ('49).

Clinical and biochemical aspects

Clinical and biochemical laboratory observations were made at regular intervals as follows: *(a) Blood serum* with respect to total protein (Ma and Zuazaga, '42), albumin (Howe, '21a, b), non-protein nitrogen (Daly, '33), cholesterol esters (Sperry and Brand, '43); *(b) blood,* for hematocrit, hemoglobin, red and white cell counts (Todd and Sanford, '43); and *(c) urine* with respect to albumin, which was determined qualitatively on random samples.

The determinations of total nitrogen in the food specimens, three-day feces, and three-day urine collections were per-

formed by the micro-Kjeldahl method of Ma and Zuazaga ('42).

RESULTS AND CONCLUSIONS

There were no significant changes in body weight, hemoglobin, serum proteins, blood count, cholesterol, non-protein nitrogen, or systolic or diastolic blood pressure.

On the basis of the results presented in table 3, certain conclusions may be drawn concerning nitrogen absorption and nitrogen balance:

TABLE 2

Effect of chemically prepared anti-trypsin (ovomucoid) upon in vitro digestion of autoclaved commercial egg white powder

DAY OF DIGESTION	HEATED EGG WHITE	HEATED EGG WHITE PLUS ANTI-TRYPSIN
	Titration units	
1	2.58 (19.8%)[1]	— 0.40
2	4.02	— 0.06
4	4.55 (17.5%)[1]	— 0.13
7	5.15 (15.0%)[1]	0.55

[1] Values in parentheses refer to per cent digestion of substrate per day calculated by the Schultz rule, as in table 1.

(a) As measured by three-day fecal nitrogen, unheated commercial egg white powder was absorbed to a somewhat lesser extent than the cooked form, or the cooked form plus ovomucoid; however, its absorption was not markedly less than that for lactalbumin.

(b) A more negative nitrogen balance occurred with unheated commercial egg white powder than when the heated form was used, or when ovomucoid was added to heated egg white; however, the nitrogen balance from the unheated egg white powder was in turn less negative than that obtained when a corresponding amount of lactalbumin was used.

(c) When ovomucoid was isolated from other proteins of egg white and then fed in proportionate amounts with heated dried egg white, there were only negligible (if any) adverse effects upon absorption of the egg white and upon nitrogen

TABLE 3

Nitrogen balance (average by three-day sub-periods) for two subjects during feeding with supplements of lactalbumin, unheated egg white, heated egg white, and heated egg white plus ovomucoid

	PERIOD [1]				
	I	II	III	IV	V
A. Supplement					
Type of protein	Lactalbumin unheated	Egg white unheated	Egg white heated	Egg white heated (a) ovomucoid (b)	Lactalbumin unheated
% of total N intake accounted for by N of supplement	41.2	40.8	41.2	38.0 (a) 7.7 (b)	41.2
B. Diet					
1. Basal diet, gm N	13.62	13.80	13.56	13.62	13.62
2. Test proteins, gm N	9.54	9.51	9.51	9.51	9.54
3. Ovomucoid, gm N	0	0	0	1.92	0
4. Total diet, gm N	23.16	23.31	23.07	25.05	23.16
C. Excretion					
1. Feces, gm N					
(a) Subject 1	6.30	7.39	5.92	5.97	7.40
(b) Subject 2	4.83	5.31	3.27	4.73	4.23
2. Urine, gm N					
(a) Subject 1	23.3	19.3	18.3	19.5	22.8
(b) Subject 2	21.2	20.1	19.2	20.5	18.8
D. Absorption					
1. Absorption, gm N					
(a) Subject 1	16.86	15.92	17.15	19.08	16.84
(b) Subject 2	18.33	18.0	19.8	20.32	18.93
2. Absorption, % of intake					
(a) Subject 1	72.8	68.3	74.3	76.2	73.1
(b) Subject 2	79.1	77.2	85.8	81.1	81.7
E. Retention					
1. Nitrogen retained, gm N					
(a) Subject 1	−6.44	−3.38	−1.15	−0.42	−7.04
(b) Subject 2	−2.87	−2.10	+0.60	+0.18	+0.13

[1] Each period lasted 9 days except period V, which lasted 6 days.

retention, as measured by nitrogen excretion in feces and urine.

(d) Therefore, it follows that there can be no direct application to human feeding programs of results obtained from *in vitro* studies of the digestibility of unheated commercial egg white powder. It is concluded that egg white anti-trypsin exerts very little deleterious effect upon nitrogen balance in man under the conditions herein described.

(e) As compared with lactalbumin, heated dried commercial egg white powder was a protein of high biologic value when used as a supplement to the basal diet.

DISCUSSION

Vernon ('04) first reported that fresh egg white contained an anti-tryptic substance. Thereafter, Falta ('06), Hamalainen and Helme ('07), Cathcart and Green ('13), Mendel and Lewis ('13), Bateman ('16) and others described many of the effects found upon feeding raw egg white to animals. The anti-tryptic activity of egg white has been attributed variously to: (a) the resistance of the egg white proteins to digestion, and preferential absorption of egg white on the enzyme; (b) the egg white globulins; and (c) a hydrolysis product of egg white proteins. Concerning the chemical nature of egg white anti-trypsin little was known until Balls and Swensen ('34) reported that it contains nitrogen and has an optical rotation similar to that of ovomucoid. Meyer and co-workers ('36) indicated that a highly active anti-trypsin preparation of Swensen's had the properties and composition of an egg mucoid. It remained for Lineweaver and Murray ('47) to show that the anti-tryptic activity of egg white appeared quantitatively in its mucoid fraction, that ovomucoid could not be fractionated into components of high and low anti-tryptic activity, that electrophoresis failed to reveal the presence of another protein in ovomucoid, that less than one molecule of ovomucoid was necessary to produce 50% inhibition of one molecule of trypsin, and that heat-denatured ovomucoid had little or no anti-tryptic activity. It

was thus concluded that egg white anti-trypsin was identical with ovomucoid.

Ovomucoid is a relatively stable protein. While anti-tryptic activity is destroyed by heat, this denaturation takes place at a rate much slower than that for most proteins. Lineweaver and Murray ('47) point out that in an aqueous solution, at pH between 3.0 and 7.0, heating at 80°C. for 30 minutes produces no essential destruction of anti-tryptic activity, but at pH 9.0 at the same temperature all the activity is lost. Boiling an egg for three minutes results in a loss of 35% of the activity, while 75% is lost in an egg boiled for 10 minutes. In the dry form, inactivation takes place more slowly, but heating for 18 hours at 60°, 110°, and 140°C. causes activity losses of 40, 94, and 100%, respectively.

It is well known that the application of heat to many proteins changes "digestibility." In most instances this is due to a partial digestive effect of the heat *per se*. However, where an anti-tryptic factor is present, such as occurs in egg white or in soybeans and other legumes, this too is destroyed by heat. The proportionate effects of heating *per se* and the removal of anti-trypsin are not known. Consequently, human feeding studies of raw versus heated egg white of the type reported by Rose and McLeod ('22), or of raw varsus autoclaved soybean as reported by Lewis and Taylor ('47), while providing valuable data on the over-all effect of heat on these raw foods, do not clearly delineate the significance of their respective anti-tryptic substances. It was for this reason that the design of the present experiment included one period when a proporionate amount of chemically isolated ovomucoid was added. It was necessary, of course, to determine that no change had taken place in the anti-tryptic character of the ovomucoid, and this was done by *in vitro* testing as is shown in table 2.

From the results of the feeding tests it appears that although unheated commercial egg white may have considerable anti-tryptic activity *in vitro*, yet it does not seem to have any markedly deleterious effect upon nitrogen retention by adults.

These findings are similar to those reported by Lewis and Taylor ('47), who fed raw and autoclaved soybean as the sole source of dietary protein. For these reasons it is apparent that the results of *in vitro* testing of the digestibility of foods containing anti-tryptic substances cannot be applied to human feeding programs without additional testing in man. In the case of unheated commercial egg white powder we have an example of a protein which on the basis of *in vitro* testing would be expected to have a low biologic utilization value, yet actually it was found to be somewhat superior in this respect to one of the widely accepted standard proteins (lactalbumin) when tested by nitrogen balance measurements.

SUMMARY

1. A study was conducted of the effects of ingested anti-tryptic factor upon nitrogen balance in man.

2. Two subjects living in a well-standardized hospital environment consumed daily a standard diet containing 2,500 Cal. and approximately 50 gm of protein. About 40% of the protein intake was comprised of a test protein during each of 5 periods, as follows: Period 1, lacalbumin (9 days); period 2, unheated commercial egg white powder (9 days); period 3, heated commercial egg white powder; period 4, as in period 3, plus a small quantity of chemically isolated ovomucoid, the egg white anti-tryptic factor (9 days); period 5, lactalbumin (6 days).

3. There were only minor differences in the amounts of nitrogen absorbed during these periods. Nitrogen retention was least favorable in the lactalbumin periods, and it appeared that chemically prepared ovomucoid had no unfavorable effect upon nitrogen utilization. Unheated commercial egg white, while less well utilized than the heated form, was nevertheless better than lactalbumin in this respect. It was concluded that egg white anti-trypsin exerts very little deleterious effect upon nitrogen balance in man under the conditions of the present experiment.

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CALCIUM AND PHOSPHORUS REQUIREMENTS OF BREEDING BOBWHITE QUAIL

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The percentage of dietary calcium necessary for optimum reproduction in gallinaceous birds depends on the number of eggs laid, the quantity of feed consumed, and the phosphorus content of the diet (Branion, '38; Titus, '39; Ewing, '41). Eggshell formation, compared with bone calcification, is an extremely rapid process. In the case of poultry, it is known that the calcium carbonate is secreted by the glands in the wall of the oviduct, a process requiring only 12 to 16 hours (Cruickshank, '35). In the absence of a sufficient supply of calcium, egg production is lowered, with the eggshells becoming progressively thinner. The serum calcium of the hen falls and there is a depletion of the calcium and phosphorus contents of the bones. Nevertheless, according to Branion, the percentage composition of the shell remains constant, showing that, as in bone formation, if the mineral is deposited, it is laid down in the normal proportion. Deobald et al. ('36) concluded that about 10% of the calcium stored in the bones may be called upon for egg production.

Branion points out that not as much research has been done with breeding birds as with chicks. Norris and associates ('33, '34) concluded that 1.65% of calcium is sufficient to meet the requirements of laying hens as judged by the egg production, shell strength, and ash, and by the levels of calcium and phosphorus in the blood. When the calcium level of the diet was

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1.8%, a level of 0.5% phosphorus was insufficient but 0.75% was adequate. Miller and Bearse ('34) were convinced that egg production is definitely affected by the quantity of phosphorus in the diet. They obtained their highest production with 0.80%, and found that 2.25% of calcium was adequate. According to Titus et al. ('37), pullets and hens differ in calcium requirements, and a high level of calcium, such as 5.4%, adversely affects hatchability. In disagreement with Miller and Bearse, these workers concluded that the level of phosphorus intake had no effect on egg production.

There is no report in the literature of work conducted on the calcium and phosphorus requirements of breeding gamebirds. Inasmuch as the bobwhite quail *(Colinus virginianus)* is one of the most popular gallinaceous upland gamebirds in the Eastern United States, an investigation was started with this species at the Patuxent Research Refuge, Laurel, Maryland, to determine its requirements for optimum reproduction. In these studies the effects of varying dietary levels of the two minerals, and of variations in the ratios between the two, have been determined.

EXPERIMENTAL

During the breeding seasons of 1946, 1947 and 1948, paired adult quail housed in individual breeding compartments were divided into groups of from 12 to 16 pairs each. Each group received a diet designed to meet the known requirements of breeding quail for protein, fat, fiber, total energy, and the various fat- and water-soluble vitamins. The diets differed in the percentages of phosphorus present, and in the Ca/P ratios. In the first series of tests (1946), each of the 6 diets studied contained 0.75% phosphorus, while the Ca/P ratios were 1:1, 1.69:1, 2.31:1, 3.1:1, 3.79:1, and 4.48:1. The same 6 Ca/P ratios were used in the second series (1947), but the phosphorus level of all diets in this series was 1.00%. In the third series (1948), the Ca/P ratio in each of the three diets was 2.33:1, with phosphorus levels of 0.75, 1.0, and 1.25%, respectively. The composition of the diets is presented in table 1.

TABLE 1

Composition of diets

INGREDIENT	DIET NO.														
	46–1	46–2	46–3	46–4	46–5	46–6	47–1	47–2	47–3	47–4	47–5	47–6	48–1	48–2	48–3
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Ground yellow corn	50.20	48.83	47.34	45.82	44.15	42.85	47.20	45.44	43.24	41.05	38.86	36.65	44.77	43.16	41.52
Standard wheat middlings	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Soybean oil meal	22.00	22.21	22.54	22.86	23.38	23.51	24.00	24.50	25.00	25.50	26.00	26.50	25.00	25.00	25.00
Dried whey	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Liver meal	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00	11.00
Salt mix [1]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vit. A and D oil (3,000 A — 425 D)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Steamed bone meal	1.80	1.80	1.80	1.83	1.82	1.83	...	...	...	...	...	...	...	...	...
Limestone	...	1.16	2.32	3.49	4.65	5.81	...	1.25	2.94	4.62	6.30	8.00	2.55	2.45	2.75
Tricalcium phosphate	...	...	...	...	...	...	2.80	2.81	2.82	2.83	2.84	2.85	1.68	3.39	4.73
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Total Ca [2]	0.75	1.27	1.80	2.32	2.85	3.37	1.17	1.67	2.33	3.00	3.66	4.33	1.76	2.33	2.92
Total P [2]	0.75	0.75	0.75	0.75	0.75	0.75	1.00	1.00	1.00	1.00	1.00	1.00	0.75	1.00	1.25
Ca/P	1.00	1.69	2.31	3.09	3.79	4.48	1.17	1.67	2.33	3.00	3.66	4.33	2.33	2.33	2.35

[1] The salt mixture consisted of iodized common salt (NaCl) 100 gm, and anhydrous manganous sulfate ($MnSO_4$) 1.7 gm.

[2] Based upon analysis of finished diets.

Records were kept of the body weights of the birds, food consumption, egg production, fertility and hatchability. Eggs were collected three times per week and stored in a cooling shed before being incubated. No eggs were kept over two weeks before being incubated.

During the periods June 9 to 14 and July 13 to 19, 1946, all eggs were saved for chemical analysis to determine the effects of dietary variations on the calcium and phosphorus contents of the shells, shell membranes, and yolks and whites.

The results of these studies are shown in tables 2 and 3. Inasmuch as there may have been some slight differences in the genetic backgrounds of the birds, or in the management technics employed in different years, it appears inadvisable to make direct comparisons between results obtained in different seasons.

Mortality

The highest mortality rate (4.2%) occurred on the diets having the highest calcium content. However, there did not appear to be any dietary effect and the mortality rate did not appear abnormal.

Live weights

The body weights of the birds were apparently unaffected by the Ca/P ratios, or by the levels of calcium and phosphorus in the diets. The observed changes in weight were normal for breeders.

Feed consumption

The consumption of food increased directly with the increase of calcium in the diet. Exceptions to this statement may be noted in the cases of diets 48–2 and 48–3.

Egg production

In each of the first two experiments the highest egg production was obtained on diets with a Ca/P ratio of 2.33:1. When the phosphorus content of the diet was 0.75%, the differences in production between the 2.33:1 ratio and the ratios of 1:1,

TABLE 2

Results of feeding breeding quail diets varying in calcium and phosphorus content

RESULTS	DIET NO.														
	46–1	46–2	46–3	46–4	46–5	46–6	47–1	47–2	47–3	47–4	47–5	47–6	48–1	48–2	48–3
Mortality (%)	1.4	1.4	2.8	1.4	0	1.4	0	2.8	2.8	2.8	4.2	4.2	0	0	0
Av. weight change (gm)	−7	−6	−7	−11	−9	−9	+2	+3	+1	−5	−2	−2	+3	+4	+5
Av. food consumption (gm)	15.8	16.3	16.4	16.4	16.7	16.7	16.4	16.6	17.6	17.5	17.4	18.0	16.9	18.5	18.4
Av. no. eggs/hen	31	41	56	47	49	37	56	56	72	54	45	53	67	64	56
Defective eggshells (%)	27	16	7	11	12	7	16	9	10	12	6	7	2	1.4	1.2
Fertility (%)	93	87	89	86	84	92	89	83	83	85	86	79	87	92	89
Hatchability (%)	66	68	69	69	67	64	72	50	60	62	58	62	68	55	61

1.69:1, 3.79:1, and 4.48:1 are all significant statistically (odds of 99 to 1 in the case of the 1:1 ratio). However, the differences resulting when the phosphorus content of the diet was 1.00% were not significant. When the 1946 results are compared with those obtained in 1947, it appears that egg production on diets containing 1.00% phosphorus was significantly higher than that on diets containing 0.75%. However, no significant differences resulted when the two levels were tested simultaneously in 1948.

TABLE 3

Calcium and phosphorus content of eggs produced by quail fed diets of 6 different Ca/P ratios at a phosphorus level of 0.78%

COMPONENT	COLLECTION DATE	CA/P RATIO					
		1:1	1.69:1	2.3:1	3:1	3.7:1	4.3:1
Shells and shell membranes							
Calcium (%)	June 9–14	30.1	30.3	31.0	31.1	31.9	31.7
	July 13–19	18.0	26.4	23.6	30.0	30.3	33.8
Phosphorus (%)	June 9–14	0.20	0.21	0.20	0.20	0.20	0.18
	July 13–19	0.60	0.44	0.42	0.23	0.13	0.14
Yolks and whites							
Calcium (%)	June 9–14	0.22	0.23	0.21	0.31	0.30	0.30
	July 13–19	0.34	0.25	0.28	0.23	0.33	0.24
Phosphorus (%)	June 9–14	0.31	0.30	0.27	0.31	0.29	0.29
	July 13–19	0.73	0.77	0.69	0.51	0.79	0.83

Eggshell condition

In each of the first two years the percentage of defective shells (cracked, checked, rough or abnormal) was greatest on diets containing a Ca/P ratio of 1:1. The differences were less marked when the diets contained 1.00% of phosphorus.

Egg fertility

Fertility was unaffected by the Ca/P ratio, or by the levels of calcium and phosphorus in the diets.

Egg hatchability

The highest hatchability occurred on the ratios of 2.3:1 and 3:1, when the phosphorus level of the diet was 0.75%. On diets containing 1.00% phosphorus the highest hatchability occurred on the Ca/P ratio of 1:1. However, there were no significant differences in any of the experiments.

Calcium and phosphorus content of eggs

The results of chemical analyses on eggs collected during the weeks of June 9 to 14 and July 13 to 19, 1946, are presented in table 3. The average calcium content of the yolk and white of all eggs, regardless of the diet, was 0.27%, or 0.02 gm per egg, and that of the shell and shell membrane was 29.17%, or 0.94 gm, making the calcium content of an entire egg weighing approximately 9.0 gm, 10.7%, or about 1.00 gm.

Neither the Ca/P ratio of the diet nor the period of the season during which the eggs were laid had any significant effect on the calcium content of the yolks and whites. During the early part of the laying season the Ca/P ratio of the diet had only a slight effect on the percentage of calcium in the shell and shell membrane, but a significant effect was noted later in the season. The calcium content of the shells from birds on the 1:1 ratio dropped 40.0%, and that of the shells from birds on the 4.33:1 ratio increased 6.0%.

The average phosphorus content of the yolk and white of all the eggs was 0.50% and that of the shell and shell membranes, 0.26%, or 0.008 gm, making the total phosphorus content of the egg approximately 0.05 gm.

During the week of June 9 the phosphorus content of the eggs was unaffected by the variations in the calcium content of the diet. However, during the week of July 13 the phosphorus content of the yolks and whites was double that of the previous period, but apparently was unaffected by the level of dietary calcium. There was a reverse correlation between the phosphorus content of shells and shell membranes collected during the later period and the level of dietary calcium. When the

Ca/P ratio in the diet was either 1:1, 1.67:1, or 2.3:1, the phosphorus content of the shells and shell membranes was significantly higher in July than in June, whereas when the ratio was greater than 3:1 the reverse situation was noted.

DISCUSSION

As a result of their studies with chickens, Titus and associates ('37) developed an equation for estimating the percentage of calcium required in the diet for any given rate of egg production. This equation, modified for bobwhite quail, is expressed as follows:

$$Ca = \frac{1.292\ (PF - 5.0\ E) + 100\ E}{F},$$

in which Ca = the required percentage of calcium in the diet; 1.292 = the ratio of calcium to phosphate in di-calcium phosphate, the form in which phosphorus appears in the diet; P = the percentage of phosphorus in the diet; F = the number of grams of feed consumed by a laying quail during the year (approximately 5475 gm); 5.0 = 100 times the quantity of phosphorus (0.05 gm) in the average egg; E = the number of eggs produced in one year (for quail in captivity, an average of 60 eggs); and 100 = 100 times the quantity of calcium (1.00 gm) in an average egg.

With the phosphorus level at 0.75%, the optimum level of calcium in the diet should be 2.0% according to this equation. The actual levels of calcium tested which were nearest this quantity were 1.8 and 2.3%. The highest egg production occurred on the 1.8% level (Ca/P ratio of 2.33:1), but the difference between it and production on the 2.3% level was not statistically significant. Similarly, the highest hatchability of eggs occurred on these two levels.

With the phosphorus level at 1.00%, the optimum level of calcium in the diet should be 2.32% according to the equation. In the second experiment the highest egg production occurred on the calcium level of 2.33%. Therefore, in the first two experiments at least, the actual results tend to agree with the

calculated value for the optimum level of dietary calcium when the phosphorus is maintained at either 0.75 or 1.00%.

Branion ('38; see also Ewing, '41) was skeptical of the worth of the equation on the ground that it does not make full allowance for variations in the rate of production or size of egg. Inasmuch as any such formula is based upon the flock average, its use for incorporating a fixed quantity of mineral into a diet will cause high-producing hens to be underfed and low producers, overfed. While Branion's objection probably is valid for chickens whose range in production and egg size may be great, it should not be very important in the case of quail. The latter lay eggs that vary little in weight and seldom number more than 100 per breeding season for birds in captivity.

From these data it would appear that the calcium and phosphorus requirements of breeding quail may be supplied by diets containing 0.75% phosphorus and 1.8% calcium (Ca/P ratio of 2.33:1). No significant change in the condition of the breeders, egg production, fertility, hatchability, or survival of offspring during the first 5 days after hatching resulted when the phosphorus level in the diet was increased. The ratio of calcium to phosphorus appears quite important in diets containing 0.75% phosphorus, but the effects of changes in the Ca/P ratio are less marked when the experimental diets contain 1.00% phosphorus.

However, observations made in the course of other studies during the winter of 1948–49 indicate that the criteria used in the evaluation of results may have been inadequate. All chicks hatched during the 1948 season were reared on a normal diet, and were divided into groups of 20 birds each on November 1. Distribution into groups was on the basis of the summer diets of the parents of the experimental birds, in that the parents of approximately one-third of the birds in each group had been fed diet 48–1 (0.75% phosphorus); the parents of an equal number had received diet 48–2; while the parents of the remaining birds had received diet 48–3. The diets fed during the winter supplied adequate amounts of protein, fat, carbohydrate

and minerals, but varied in their contents of vitamin A, vitamin K, and the various members of the vitamin B complex. The only known differences among the experimental birds were the dietary backgrounds of the parents. It was found that 23.6% of the chicks from parents fed diet 48–1 (0.75% phosphorus, 1.76% calcium) died during the 20-week experimental period, and that the mortality rates from diets 48–2 and 48–3 were 13.3% and 8.5%, respectively. On autopsy, blood clots were found in the viscera or brain of 27% of the birds from parents fed diet 48–1, in 17% of the birds from diet 48–2, and in 15% of the birds from diet 48–3.

Presumably, these differences in the winter mortality rates of the young birds may have resulted from the differences in the diets fed the parents during the breeding season. If this is the case, it may be concluded that while a diet containing 0.75% phosphorus and 1.8% calcium is adequate to permit reproduction at a normal rate, it is inadequate to insure survival of the offspring during the winter months. As judged by the criteria of the survival and weight maintenance of the parent birds, by egg production, fertility, hatchability and survival of the offspring, it appears that breeding bobwhite quail require diets furnishing approximately 1.00% phosphorus and 2.3% calcium.

SUMMARY

In the course of studies designed to determine the calcium and phosphorus requirements of breeding bobwhite quail, it was found that best results were obtained when the Ca/P ratio in the diet was approximately 2.3:1. Variations in the Ca/P ratio produced significant differences in results when the level of phosphorus in the diet was 0.75%, but the differences were less marked when the level of phosphorus was increased to 1.00%.

Although diets containing 0.75% phosphorus and 1.8% calcium appeared adequate for reproduction, as judged by the criteria of the maintenance of satisfactory condition in the breeders, egg production, fertility, hatchability and survival

of offspring during the first 5 days after hatching, it was found that the winter mortality of the offspring of birds fed such a diet was much greater than that occurring in the offspring of birds fed on diets containing 1.00 or 1.25% phosphorus. It is concluded that breeding bobwhite quail require diets furnishing approximately 1.00% phosphorus and 2.3% calcium.

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SUCCINYLSULFATHIAZOLE AND A RAT GROWTH FACTOR IN LIVER [1]

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In connection with another investigation it was noted that rats on a purified diet containing synthetic pteroylglutamic acid (PGA) and 5 gm of succinylsulfathiazole (sulfasuxidine) per 100 gm of diet did not grow [2] so well as rats on the same diet but with the sulfasuxidine and PGA omitted. This difference in growth (weight gain) was not large, but it was consistent. These few observations led to a more detailed study of the factors concerned with this difference. The results of this study are given below.

EXPERIMENTAL

Albino rats, raised in the laboratory, were used as experimental animals. For the most part, the animals were started on the experimental diets at about 25 days of age, and the duration of the experiments was 6 weeks. Only those litters which had as many animals of one sex as there were groups in an experiment were used. Each group was composed of one animal from each of 6 or 7 litters and all animals of any

[1] Most of the data presented here were collected during 1946 and the first three months of 1947, but the completion of the experimental work and preparation of the manuscript were unavoidably delayed until recently.

[2] Measured as individual weights only.

one litter were of the same sex.[3] The animals were kept in individual cages and weighed daily. The diets were prepared in small amounts and stored in closed containers in the cold room until used or discarded. In the first two of the three experiments reported here, total white cell and differential counts and hemoglobin determinations were made during the first three weeks of the experiments and again during the second three weeks. These determinations were usually made toward the end of each of the two periods. Only the counts taken during the second three weeks are reported here.

Experiment 1

There were 6 groups of animals in the first experiment. The diets used are given in table 1. The casein of these diets was extracted in the laboratory by cold percolation with about 6 changes of the extracting fluid per week. The casein was first extracted with 95% ethyl alcohol, then 80% alcohol, and finally 50%. In each case the extraction was continued until the effluent was nearly colorless.

The liver preparation [4] was the 70% alcohol soluble fraction of the material obtained from hog liver by hot water extrac-

[3] When this system of distribution is used, any two animals of the same litter can be considered a pair, and any two groups within a given experiment can be compared statistically by determining the standard error of the differences in weight gains between the two members of all pairs that make up the two groups.

Thus,

$$\sqrt{\frac{\Sigma(d^2)}{(N-1)N}} = \text{Standard error, and}$$

$$\frac{\text{Mean of the differences}}{\text{Standard error}} = \text{"t,"}$$ where d is the deviation of each difference from the mean of the differences and N is the total number of pairs.

This method of distribution of the animals, when growth is the criterion, and also this procedure for the calculation of the degree of significance, are not only highly sensitive but they decrease the error when males and females are used in the same experiment.

The difference between two means was considered significant if the probability that the results were due to chance was 0.05 or less.

[4] The authors wish to thank Parke, Davis and Co., Detroit, for the crude liver extract; Merck and Co., Inc., Rahway, N. J., for most of the B vitamins; Lederle Laboratories, Inc., Pearl River, New York, for the pteroylglutamic acid; and Sharp and Dohme, Inc., Glenolden, Pa., for the succinylsulfathiazole and antipernicious anemia liver concentrate.

tion which represented 50 gm of fresh liver per gram of extract.

The first group of animals of experiment 1 was given the basal or no. 1 diet, i.e., the PGA, sulfasuxidine and liver extract were all omitted. All other groups received this diet plus one or more supplements. The second group received sulfasuxidine, while groups 3, 4, and 5 were given sulfasuxidine and

TABLE 1

Diets used in experiment 1

INGREDIENTS [1]	GROUP NO.					
	1	2	3	4	5	6
Casein (alcohol ext.)[2] (%)	18	18	18	18	18	18
Salt mixture [3] (%)	4	4	4	4	4	4
Cottonseed oil (%)	2	2	2	2	2	2
Cellulose [4] (%)	2	2	2	2	2	2
Liver extract [2] (%)	..	..	..	..	..	2
Sucrose (%)	74	74	74	74	74	72
PGA [5] (mg %)	..	..	30	60	120	..
Sulfasuxidine [6]	..	+	+	+	+	+

[1] In addition, each diet contained the following vitamins in mg %: Thiamine chloride, 0.5; riboflavin, 0.5; pyridoxine, 0.5; calcium pantothenate, 3.0; *i*-inositol, 3.0; nicotinic acid, 3.0; 2-methyl-1,4-naphthoquinone, 0.025; choline chloride, 200. Approximately 0.5 ml of cod liver oil was given by mouth to each animal twice weekly and 0.4 μg of biotin by intraperitoneal injection three times per week.

[2] See text.

[3] Jones and Foster ('42).

[4] Cellu flour.

[5] Pteroylglutamic acid.

[6] Succinylsulfathiazole; 5 gm per 100 gm of diet.

varying amounts of PGA (table 1), and group 6 was given sulfasuxidine and 2% of the liver extract. It was thought advisable to give the PGA at more than one level in the event that the large amount of sulfasuxidine would cause a partial inactivation or incomplete absorption of this vitamin.

The biotin used in the first experiment was made up as a concentrated solution in alcohol at the beginning of the experiment. A small portion was diluted with water at approximately weekly intervals. To determine if the results in respect

to growth were due to a decrease in activity of the biotin stock solution, it was analyzed for the vitamin at the end of the experiment. There was no detectable loss.

Results. Weight gains and white cell counts are given as averages in table 2. Most of the animals of group 2 (sulfasuxi-

TABLE 2

Initial weights, weight gains and white cell counts of experimental animals

GROUP AND DIET NO.	AVERAGE INITIAL WEIGHT	AVERAGE WEIGHT GAIN	AVERAGE WHITE CELL COUNT
	gm	*gm*	
	Experiment 1, 6 males, 1 female in each group		
1 [1]	49.1	150.1	18,200
2	50.6 [2]	56.9	8,800
3	51.4	125.6	18,900
4	50.8	115.9	19,200
5	50.8	119.9	18,300
6	50.2	153.1	16,700
	Experiment 2, 2 males, 5 females in each group		
7 [3]	44.3	136.7	16,000
8	44.4 [2]	46.6	7,400
9	44.4	107.7	17,400
10	44.4	138.0	18,000
	Experiment 3, 6 males in each group		
11 [4]	51.5	156.7	
12	51.2 [2]	48.2	
13	51.5	128.2	
14	50.5	120.3	
15	50.8	160.2	

[1] See table 1 for diets used in first experiment.

[2] As most of the animals in this group lost weight toward the end of the experiment, the maximum weight attained was used for calculating the weight gain.

[3] The diets used in experiment 2 were the same as diets 1, 2, 4 and 6 of experiment 1 with the following changes: The amounts of thiamine chloride, riboflavin, pyridoxine, calcium pantothenate, *i*-inositol, nicotinic acid, and 2-methyl-1,4-naphthoquinone were doubled and biotin was given in the diet at a level of 20 μg per 100 gm.

[4] Diets 11, 12, 13 and 15 differed from diets 7, 8, 9 and 10, respectively, only in that Labco casein instead of alcohol extracted casein was used and one drop weekly of vitamin D-enriched percomorph oil was substituted for the cod liver oil. Group 15 was given, by subcutaneous injection, 0.02 ml of Sharp and Dohme antipernicious anemia liver preparation instead of the crude liver extract, and the sucrose was increased to 74%. Diet 14 was the same as diet 13 except that the PGA was given intraperitoneally (10 μg per day).

dine supplement), and of the corresponding groups of experiments 2 and 3, gained weight at first but lost during the latter two or three weeks of the experiment, and a few died. For these animals the maximum weight attained was used in calculating the gain in weight. In experiment 1, two animals of group 2 died, but in both cases death occurred after the second blood count had been made.

As to gains in weight, table 2 shows that (a) the animals of group 1 (basal) grew very well, (b) those of group 2 (sulfasuxidine supplement) grew much less than any of the others, (c) the animals of groups 3, 4 and 5 (sulfasuxidine plus graded amounts of PGA) grew essentially the same as each other but definitely less than the controls, and (d) group 6 (sulfasuxidine plus liver extract) grew fully as well as the controls.

The weight data were analyzed statistically according to the method described in footnote 3 and the results are presented in table 3. Here are given the "t" values obtained from the statistical comparison of each group with every other group in an experiment. The difference in weight gain between groups 1 and 6 was not significant, but these two groups grew significantly more than any of the others; there was no real difference among groups 3, 4 and 5, but these all grew significantly more than the animals of group 2. As there was essentially no difference among groups 3, 4 and 5, it would appear that even the lowest level of PGA was sufficient to produce the maximum effect possible for this vitamin.

The white cell counts (table 2) were very nearly the same for the animals of the control group and of the three groups receiving PGA, while for group 6 they were slightly reduced and for group 2 they were about one-half those of the others. The neutrophils were still further reduced in the animals of group 2 and to a less extent in those of group 6. The percentage of this class of leukocytes for each of the 6 groups was 18, 9, 16, 20, 20 and 13, respectively. None of the animals showed any evidence of reduced hemoglobin.

Experiment 2

The probability existed that the large amount of sulfasuxidine may have partially destroyed some of the vitamins or prevented their complete absorption. Experiment 2 was designed to test this probability. There were 4 groups in this

TABLE 3

"t" values for all possible comparisons within each experiment

GROUP NO.	1	2	3	4	5	7[2]	8	9	11	12	13	14
2	7.94[1]											
3	3.29	6.77										
4	3.96	4.96	1.39									
5	5.42	4.94	0.81	0.42								
6	0.40	6.35	3.70	4.01	9.10							
8						11.0						
9						7.80	6.99					
10						0.17	7.28	4.85				
12									8.11			
13									2.48	9.67		
14									3.73	6.57	3.11	
15									0.071	20.9	4.19	3.84

[1] The "t" value for the 0.05% point for 6 degrees of freedom is 2.445 and for 5 degrees of freedom is 2.571.

[2] The different experiments are separated by double lines.

experiment (groups 7, 8, 9 and 10), corresponding to groups 1, 2, 4 and 6 of experiment 1. As it was shown above that the slower growth on the part of the animals receiving the sulfasuxidine and PGA was not due to insufficient PGA, only one level of this vitamin was included in the second experiment. The diets used for groups 7, 8, 9 and 10 differed from those of the corresponding groups of experiment 1 in that the

amount of thiamine chloride, riboflavin, pyridoxine, calcium pantothenate, *i*-inositol, nicotinic acid and 2-methyl-1,4-naphthoquinone was doubled, and biotin was included (20 μg per 100 gm of diet).

The results of this experiment, shown in tables 2 and 3, are very much the same as those of the first experiment. Here again the animals of all other groups gained significantly more weight than the animals of group 8, while groups 7 and 10, which were nearly identical, gained significantly more than group 9.

One of the animals of group 8 died before the white cells were counted the second time, so the value for this group is the average of 6 observations. In general, the results obtained with the white cell counts were the same as in the first experiment. Two of the animals of group 8 were in a moribund condition when the counts were made and the neutrophil percentages were very high. This high count would indicate that these animals had an infection, but none was observed. Omitting these two animals, the average per cent of neutrophils for each of the 4 groups of experiment 2 was 14, 6, 18 and 11 respectively. Here again hemoglobin levels were essentially normal.

In the first experiment the animals receiving sulfasuxidine and liver extract had a slightly lower white cell count and a considerably lower neutrophil percentage than any of the groups receiving pteroylglutamic acid, and in the second experiment the total white cell count of the group receiving the liver extract was as high as the PGA group but the neutrophils were lower in the former than in the latter. Apparently the liver extract contained sufficient pteroylglutamic acid to produce good growth but not enough to give the same leukocyte picture as appeared in the animals receiving the synthetic compound.

Experiment 3

The purpose of experiment 3 was to study the effect on growth of an antipernicious anemia liver concentrate in place of the crude liver extract, and to determine whether injecting

the PGA instead of giving it with the diet would allow the sulfasuxidine further to decrease growth. The basal or control diet was the high vitamin diet of experiment 2, except that vitamin-free casein [5] instead of the alcohol extracted casein was used and percomorph oil, one drop per week, was substituted for the cod liver oil. There were 5 groups of animals, the first three of which (11, 12 and 13) were fed diets the same as those fed groups 7, 8 and 9 of experiment 2; that is, basal, basal plus sulfasuxidine, and basal plus sulfasuxidine and 60 μg % of PGA. Group 14 was given, in addition to the control diet, sulfasuxidine in the diet and 10 μg of PGA per day by intraperitoneal injection. Group 15, in addition to the basal diet plus sulfasuxidine and 60 μg % of PGA, was given subcutaneously 0.02 ml of antipernicious anemia liver concentrate [6] instead of the crude liver extract. White cell counts and hemoglobin determinations were not made on the animals of experiment 3.

The results of this experiment are also given in tables 2 and 3. Here again the average gains in weight of all other groups of animals were significantly more than those of group 12; the gains of group 11 just missed being significantly more than those of group 13, but the differences between 11 and 14 and 13 and 14, as well as between 15 and 14 and 15 and 13, were significant. None of the other pairs showed a real difference. It is to be noted that the average gain in weight for the group with the PGA in the diet was 8 gm more than for the group in which the PGA was given intraperitoneally. Although this difference is not great it was constant among the various pairs of animals, which accounts for the fairly high degree of significance. But the most striking result of this experiment was the excellent growth produced by the small amount of the antipernicious anemia preparation.

DISCUSSION

Nearly two decades ago Guha ('31) reported that milk contained a heat labile factor that increased the growth of young weanling rats when added to a diet of maize, wheat, "light

[5] Labco.
[6] Sharp and Dohme.

white'' casein, powdered yeast, $CaCo_3$ and NaCl. Sheep liver was less effective than milk. The following year Mapson ('32) observed that the addition of ox liver to a synthetic diet containing yeast produced larger rats than the same diet without the liver. White and Sayers ('42) obtained similar results using protein of pancreas. Hartman and Cary ('42) found that milk contained a growth promoting factor in rats when the B factors were supplied by yeast or yeast supplemented with all of the then known B vitamins, vitamin E, and vitamin K. Jaffé ('46) and Jaffé and Elvehjem ('47) grew rats on a diet of natural foods; here again growth was stimulated by liver or a laboratory prepared extract of fresh liver. The latter workers supplemented their diet of natural foods with synthetic B factors. They also obtained similar results with a synthetic diet. Sporn, Ruegamer and Elvehjem ('47) have demonstrated that rats born of females on a synthetic diet grow more slowly and have a higher mortality than the young born of dams on the same diet supplemented with liver.

More recent investigators who have made comparable observations are Bowland, Ensminger and Cunha ('48) and Zucker and Zucker ('48). The latter authors have emphasized the association of this growth factor with animal proteins.

Numerous reports have emphasized the need of the chick, the mouse, and certain bacteria for a nutrient found in several materials but especially in liver.

Finally, Rickes et al. ('48) succeeded in crystallizing a factor which they termed vitamin B_{12}. It has been shown that this crystalline compound is beneficial in Addisonian pernicious anemia (West, '48), and promotes the growth of *Lactobacillus lactis* (Shorb, '48), chicks (Ott et al., '48; Lillie et al., '48), rats (Emerson, Wurtz and Zanetti, '49), and pigs (Johnson and Neumann, '49; Hogan and Anderson, '49).

In most of the experiments referred to above in which purified casein was used as the protein, growth was poor unless the growth factor was added. In the experiments reported in the present paper, the growth of animals fed the purified protein was nearly as good as those receiving the sulfasuxidine

plus liver. The casein used in the first two experiments may not have been sufficiently purified, but in the last experiment vitamin-free casein was used.[7] Here again the average gains in weight of the animals receiving the liver extract and of the animals on the basal diet differed by only 3.5 gm. Hartman and Cary ('42) and Sporn et al. ('47) found this growth factor in milk, Mapson ('32), Sporn et al. ('47) and Bosshardt et al. ('49b) reported that it could be stored in the animal body, and Mapson showed that the factor could be carried through the mother's milk. Fresh milk is given ad libitum to our stock animals, and the young used in the experiments reported here were continued on the same regimen until they were put on experiment at 25 days of age. They undoubtedly had considerable stores of the necessary growth factor, which would explain the good growth of the animals on the basal diet.

It is impossible to conclude with finality that vitamin B_{12} is the factor in liver which is responsible for the increased growth of our animals receiving the sulfa compound. However, since only 0.02 ml of the antipernicious anemia concentrate produced excellent growth in animals receiving PGA and sulfasuxidine, it is very possible that such is the case.

The mechanism by which succinylsulfathiazole increased the need for the growth factor is unknown at the present time. The most logical explanation is that intestinal bacteria form small amounts of this factor, and that the bacteria are inhibited by the sulfa drug from doing so. If that is the case, succinylsulfathiazole must have the power to produce bacterial stasis in the presence of pteroylglutamic acid.

As this manuscript was being prepared an abstract of a paper by Bosshardt et al. ('49a) appeared, reporting results with mice comparable to those presented here using rats.

SUMMARY

Young rats on a synthetic diet containing purified casein and all members of the vitamin B group except pteroylglutamic acid and vitamin B_{12} grew well. The addition of succinylsulfa-

[7] See footnote 5, page 586.

thiazole produced the usual results, namely, failure of growth, leukocytopenia with the granulocytes affected more than the other cells, and finally death.

The inclusion of pteroylglutamic acid in the succinylsulfathiazole diet corrected the blood dyscrasia and prevented the death of the animals but failed to restore growth completely. Increasing the amount of pteroylglutamic acid did not change the picture.

Animals given succinylsulfathiazole plus a crude liver extract had normal blood and grew at a rapid rate.

Doubling the amount of 2-methyl-1,4-naphthoquinone and of the dietary B vitamins except choline did not increase the growth of rats given succinylsulfathiazole and pteroylglutamic acid.

The subcutaneous injection of 0.02 ml of a purified antipernicious anemia liver preparation in rats on the latter regimen produced very good growth.

The probable relation of this growth factor found in liver to vitamin B_{12} is discussed.

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OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of $1000.00 established by the Nutrition Foundation, Inc. for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojurning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1950 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 15, 1950.

H. E. CARTER
University of Illinois
Urbana, Illinois

CHAIRMAN, NOMINATING COMMITTEE

BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1950 Award of $1000.00 and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the judges may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1950. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1950. The nominations should be accompnaied by such data relative to the nominee and his research as will facilitate consideration for the award.

L. A. MAYNARD
Cornell University, Ithaca, New York

CHAIRMAN, NOMINATING COMMITTEE

MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1950 Award of $1000.00 established by Mead Johnson and Company to promote researches dealing with the B-complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1950.

The Award will be given to the laboratory or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year, January 1 to December 31, the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1949 must be in the hands of the Chairman of the Nominating Committee by January 15, 1950. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

W. H. Sebrell, Jr.
Experimental Biology and Medicine Institute
National Institutes of Health
Bethesda, Maryland

Chairman, Nominating Committee

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